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【物件名】　外国語図面　1

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【プルーフの要否】　要

【書類名】　外国語明細書

1　Title of Invention　Method to utilize 5' ends of transcribed regions for cloning and analysis

2　Claims

1.　A method to prepare nucleic acids tags corresponding to the 5' end of transcribed regions said mRNA.

2.　A method according to claim 1 where concatamers of such 5' end tags are produced.

【請求項２】　A method in which such 5' end specific sequence tags derived from transcribed regions said mRNA are analyzed by sequencing.

【請求項３】　The method for preparing concatemers of a plurality of at least two or more nucleic acid fragments having information on nucleotide sequences of 5' end regions of a plurality of nucleic acids related to transcribed in a sample, comprising

A first step of selectively collecting a plurality of cDNAs containing regions complementary to 5'-end regions of mRNAs, which cDNAs are formed by using RNA or mRNAs derived from a biological sample or in vitro synthesized RNA derived from cDNA - or tag - libraries in the sample as templates;

A second step to collecting fragments containing cDNA regions including at least the regions corresponding to the 5'-end regions of said mRNAs or cDNA;

And a third step of creating a concatamer of such 5' end nucleic tags.

【請求項４】　The method as in claim 4 but in which

The first step is substituting the cap-structure of mRNAs with an oligonucleotide;

The second step constitute in the formation of full-length cDNA;

The third step involve cleavage of a 5' end tag and formation of concatamers.

【請求項５】 The method according to claim 4, wherein said first st ep comprises the steps of synthesizing the first-strand cDNAs using mRNA s as templates; attaching a selective binding substance to the cap struc tures of said mRNAs; cleaving single-stranded RNAs; binding said selecti ve binding substance to a corresponding selective binding substance immo bilized on a support, which corresponding selective binding substance se lectively binds to said selective binding substance; and recovering said cDNA.

【請求項６】 A method as in claim 4 where the first step to isolate the full-length cDNA includes an RNase digestion step followed by treat ment with an immobilized cap-binding substance followed by eluting such full-length cDNAs.

【請求項７】 A method to add a sequence connected to the 5' end a nucleic acids corresponding to the 5' terminal part of a transcript, whe n such that can be recognized by a substance that is capable of cleaving such nucleic acids outside the recognition sequence.

【請求項８】 The method according to claim 4, wherein said selectiv e binding substance is biotin, and said corresponding selective binding substance is avidin, streptavidin or an avidin or streptavidin derivativ e which specifically binds to biotin.

【請求項９】 The method according to the claim 4 where the selectiv e binding substance is digoxigenin and said corresponding binding substa nce is an antibody directed against digoxigenin.

【請求項１０】 A method according to claim 4 or 9, wherein a select ive binding substance is bound to a corresponding selective binding subs tance which is immobilized on to a support, and where such a support is made of magnetic beads, agarose beads, or latex beads

【請求項１１】 The method according to any one of claims 4 and 6 to 11, wherein said second step comprises the steps of binding a linker ha

ving at least a restriction site for a substance that cleaves DNA outside its recognition sequence in the end region corresponding to the 5' end of said nucleic acids corresponding to the 5' end of genes, and a random oligomer region at the 3' end region; synthesizing a second-strand cDNA using said linker or other oligonucleotides partially or totally corresponding to the linker as a primer and said cDNA as a template; treating the obtained linker-bound double-stranded cDNA with said restriction enzyme; and selectively recovering fragments yielded by cleavage by the restriction enzyme, which fragments contain said linker moieties and part of 5' end cDNA.

【請求項１２】 The method according to any of claims 4 to 12, wherein a selective binding substance is attached to said linker; and the step of selectively recovering said fragments containing said linker moieties comprises the steps of binding said selective binding substance to a corresponding selective binding substance immobilized on a support, which corresponding selective binding substance selectively binds to said selective binding substance; and recovering said support.

【請求項１３】 The method according to any of claims 4 to 13, wherein said selective binding substance is biotin, and said corresponding selective binding substance is avidin, streptavidin, or an avidin derivative or derivatives of streptavidin which specifically binds to biotin.

【請求項１４】 The method according to any of claims 4 to 13 where the selective binding substance is digoxigenin and said corresponding binding substance is an antibody directed against digoxigenin.

【請求項１５】 The method according to any one of claims 4 to 15, wherein said restriction enzyme is a substance with a enzymatic activity to recognize nucleic acid and to cleave at a site different form the recognition site.

【請求項１６】 The method according to any one of claims 4 to 16, w

herein said restriction enzyme is a class II restriction enzyme like Gs u I , MmeI, Bpm I or Bsg I

【請求項１７】 The method using nucleic acid fragments obtained acc ording to any one of claims 1 to 18, for further comprising the steps of cloning into concatamer.

【請求項１８】 A method for determining nucleotide sequences of 5'- end regions of a plurality of mRNAs by sequencing said concatemer prepar ed by the method according to any one of claims 1 to 18.

【請求項１９】 A method, which is the same method according to any one of claim 1 to 18, except that preliminarily obtained cDNAs having co mplete length is used instead of carrying out said first step.

【請求項２０】 A method to produce 5' end nucleic acids tags corres ponding to the 5' ends of mRNA, in which a mixture of RNA molecules is p repared from a preexisting full-length cDNA library and the obtained RNA carries at the 5' end of the RNAs a sequence cleavable by a substance a ble to recognize a nucleic acids and cleave outside its recognition sequ ence.

【請求項２１】 A method to produce 5' end nucleic acids tags corres ponding to the 5' ends of mRNA, in which a mixture of nucleic acids TAG molecules is prepared from a preexisting full-length cDNA library carryi ng close to the 5' end of a sequence cleavable by a substance able to re cognize a nucleic acids and cleave outside its recognition sequence, whi ch is used to produce a nucleic acid TAG molecule.

【請求項２２】 The concatemer prepared by the method according to a ny one of claims 1 to 22.

【請求項２３】 A vector comprising said concatemer according to cla im 23.

【請求項２４】 A sequence, which is derived from a concatemer prepa red by the method according to any one of claims 1 to 22.

【請求項２５】　A method based on any of claims 1 to 22, which allows to determine the transcriptional status of a given cell and therefore the transcriptional networking.

【請求項２６】　A method, which is the same method according to any one of claims 1 to 22 to obtain expression data on a plurality of mRNA or cDNA in a sample.

【請求項２７】　A method, which is the same method according to any one of claims 1 to 22, to quantify expression data on a plurality of mRNA in a sample.

【請求項２８】　A method, which uses sequence information obtained from concatemers prepared by a method according to any one of claims 1 to 22, is used to build a database holding sequence information derived from the concatemers.

【請求項２９】　A method, which is the same method according to any one of claims 1 to 22, to identify open reading frames in a genomic sequence said genome.

【請求項３０】　A method, which is the same method according to any one of claims 1 to 22, to identify start sites of transcription and regulatory sequences upstream of the start site of transcription in a genomic sequence said genome.

【請求項３１】　A method, which uses sequence information obtained from concatemers prepared by a method according to any one of claims 1 to 22, to clone a full-length or partial cDNA from a plurality of nucleic acids.

【請求項３２】　A method, which uses sequence information obtained from concatemers prepared by a method according to any one of claims 1 to 22, to analyze the activity of regulatory regions in a genome said promoter.

【請求項３３】　A method, which uses sequence information obtained f

rom concatemers prepared by a method according to any one of claims 1 to 22, to inactivate a gene.

【請求項３４】　A method, which uses sequence information obtained from concatemers prepared by a method according to any one of the claims 1 to 22, to synthesis nucleotide sequences said linker.

【請求項３５】　A method, which uses sequence information obtained from concatemers prepared by a method according to any one of the claims 1 to 22, to synthesize nucleotide sequences said primers.

【請求項３６】　A method, which uses sequence information obtained from concatemers prepared by a method according to any one of the claims 1 to 22, to obtain extended nucleotide sequences derived from the 5'-ends of transcripts said sequencing.

【請求項３７】　A method according to any one of claims 1 to 8, wherein a single stranded cDNA is ligated two a double stranded synthetic oligonucleotide said linker, wherein the linker has a single stranded overhang encompassing a nucleotide sequence said tag, which was obtained from concatemers prepared by a method according to any one of the claims 1 to 13, wherein the linker is attached to a selective binding substance and the selective binding substance is attached to a corresponding selective binding substance said support, and where such linker bound to the support is used to enrich a specific nucleotide sequence said 1st strand cDNA said RNA transcript.

【請求項３８】　A method according to any one of claims 1 to 8, wherein a single stranded cDNA is ligated two a double stranded linker said primer, where a selective binding substance is attached to said linker, and where selectively binding substance is attached to a corresponding selective binding substance said support, and where such DNA template is used to obtain the nucleotide sequences of the 5' -region of an initial transcript said RNA.

【請求項３９】　A method based on any of the claims 1-39 to be used for the development of diagnostic tools.

3　Detailed Description of Invention

【０００１】

【発明の属する技術分野】

The present invention relates to a method to selectively collect multiple nucleic acid fragments containing information on the nucleotide sequences at the 5' end site of multiple mRNAs within a sample. The method of the present invention is effective for analyzing the mRNAs contained within the sample, for discovering new genes, and for studies on gene regulation.

【０００２】

【従来の技術】

To utilize genomic information parts of the genome are transcribed into mRNA. For the understanding of the genome and its use in regulatory processes, information on individual mRNA species is required, which should include their partial or full-length nucleotide sequence and their relative or absolute quantity in a given biological context.

【０００３】

Conventionally, the base sequences in mRNAs contained in a cell or tissue sample had been analyzed by preparing a cDNA library by reverse transcription, using mRNAs as templates and investigating the individual insert cDNA fragments within said cDNA library. Since a sample contains a large number of varied mRNAs, the conventional method is of limited efficiency to analyze gene expression profiles and to identify rare genes. Therefore other technologies have been invented to monitor the expression patterns of mRNA in complex samples and to identify genes by short sequence elements said tags.

【０００４】

High-throughput expression profiling is commonly performed by the use of so-called DNA microarrays (Jordan B., DNA Microarrays: Gene Expressio n Applications, Springer-Verlag, Berlin Heidelberg New York, 2001: Schen a A, DNA Microarrays, A Practical Approach, Oxford University Press, Oxf ord 1999). For such experiments specific probes representing individual genes or transcripts are placed on a support and simultaneously hybridiz ed with a plurality of samples. Positive signals will be obtained where a probe on the support reacts with a molecule presented with the sample. These experiments allow the parallel analysis of a large number of gene s or transcripts. However, the approach is limited to the fact that only genes or transcripts can be studied, which were initially identified by other experimental means. Such means can include cDNA libraries, partia l sequence tags and/or results obtained from computer predictions. Due t o the limitations of DNA microarray experiments alternative approaches a re in use for gene discovery and expression profiling, which are based o n partial sequences said tags obtained from a plurality of mRNA samples.

【０００５】

The so-called SAGE (Serial Analysis of Gene Expression) method is know n as an efficient method of obtaining partial information on the base se quences in mRNAs (Velculescu V.E. et at., Science 270, 484-487 (1995)). This method forms DNA concatamers by ligating multiple short DNA fragmen ts (about 10 bp) containing information on the base sequences at the 3' end site of multiple mRNAs, and determines the base sequences in these D NA concatamers. It is a method for finding out partial information on th e base sequences at the 3' end site of multiple mRNAs. When only a short base sequence close to the 3' end is available but the mRNAs itself is already known, the SAGE method can often identify the mRNA, although th e available base sequence is as short as about 10 bp. This method is cur rently in wide use as an important method for analyzing genes expressed

in specific cells or tissues.

【０００６】

【発明が解決しようとする課題】

While the SAGE method can be used to learn a partial base sequence at the 3' end site of mRNAs, it is difficult to clone new genes based on th e information in such short sequences at the 3' end site alone. Despite the application, SAGE does not teach how to obtain cDNA clones close to the 5' end of the cDNA. In fact, 4 bp restriction enzymes of class IIs a re used. A 4bp cutter usually cleaves on average a few hundred nucleotid es, which is on average $1/10^{th}$ of the average size of an mRNA transcript . Thus SAGE principles strongly suggest that 3' ends are collected with high prevalence, and no information can be collected about the 5' end fo r most of the transcript. In addition 10 bp tags have often been insuff icient for specific gene identification and mapping to genomic sequences said entire or partial genomes. Therefore, the 10 bp tags are used to i dentify only a "sage-tag", which comprises a part of a mRNA. Notice that mammalian mRNA comprises only 3-5% of the transcribed part of the mamma lian genome and the specific "sage-tag" comprises a subfraction of this 3-5%, which lies in proximity of the class IIs restriction enzyme used i n the analysis. Since a 4 bp restriction enzyme cuts approximately a ran dom sequence every 4^4 bp (256 bp), the "sage-tags" can represent approxi mately 1/256 of the 3-5% expressed fraction of the genome (calculation=l ess than 0.02%). Therefore, the SAGE techniques teach that essentially i t is not possible to use SAGE-tags to analyze a genome but only a very l imited fraction of it.

【０００７】

Accordingly, the invention claimed in this application aims to provide new means that not only enables the acquisition of information on the b ase sequences of 5'-ends in mRNAs within a sample, but also enables the

cloning of new genes and the analysis of genomic sequence information, which correspond to coding and regulatory regions.

【０００８】

This can include statistics on the DNA transcriptional starting site. By using concatamers to obtain information on a large number of 5'-sequence tags as presented in the invention, it is possible to effectively map transcriptional start sites and the related the promoter sequences. Thus the invention provides new means, where SAGE did not allow any promoter analysis due to the use of unrelated 3'-ends. At the same time, there were techniques for the collection of full-length cDNA clones and sequences derived thereof; however, those are focusing on collecting the full-length cDNA clones and not fragments covering the 5'-ends. Therefore full-length cDNA cloning approaches are not suitable for high throughput identification and analysis of start sites of transcription and the related promoter regions. The invention offers here a novel way to combine contrasting teachings and to obtain by a high throughput approach 5' ends, which are useful for promoter mapping and analysis. The use of the invention to study and analyze complex regulatory networks in combination with the ability to identify and clone new genes opens a wide area of applications for the invention to monitor biological systems and their status in development, homeostasis, and disease.

【０００９】

【課題を解決するための手段】

After devoted research, the inventors involved in this application were able to complete the present invention by arriving at the fact that by selectively collecting multiple nucleic acid fragments containing information on the base sequences at the 5' end site of the mRNAs, it is not only possible to acquire information on the base sequences in mRNAs, but it is also possible to clone new genes; and they were also able to arri

ve at a concrete method for attaining this goal.

【００１０】

That is, the present invention provides a method for preparing concatemers of a plurality of nucleic acid fragments having information on nucleotide sequences of 5'-end regions of a plurality of mRNAs in a sample, comprising a first step of selectively collecting a plurality of first-strand cDNAs containing regions complementary to 5'-end regions of mRNAs, which cDNAs are formed by using mRNAs in the sample as templates; a second step of selectively collecting fragments containing cDNA regions including at least the regions complementary to the 5'-end regions of said mRNAs; and a third step of ligating the collected fragments to form a concatemer. The present invention also provides a method for determining nucleotide sequences of 5'-end regions of a plurality of mRNAs by sequencing said concatemer prepared by the method according to the present invention. The present invention further provides a method, which is the same method according to any one of claim 1 to 10, except that preliminarily obtained cDNAs having complete length is used instead of carrying out said first step. The present invention still further provides the concatemer prepared by the method according to the present invention. The present invention still further provides a vector comprising said concatemer according the present invention. The present invention still further provides sequence tags derived form said concatemers prepared according to the present invention. The present invention still further provides means to use the sequences derived from said concatemers to analyze the content of the plurality of a RNA sample. The present invention still further provides means to use the sequences derived from said concatemers to identify regions in the genome, which are required for gene regulation and gene expression.

【００１１】

The invention is not limited to the use of concatamers for sequencing of 5' ends, but modifications at particular steps of the enrichment of 5' ends and their cloning as disclosed here allow for the individual sequencing of specific 5' ends. Such embodiments of the invention would include a modification of the first and second step, where a linker would be used that is specifically bound to a solid matrix. The cDNA bound to the support would then be used to prepare the sequencing reactions.

【００１２】

Thus the inventions refers more generally to the concept of isolating portions of nucleic acids corresponding to the 5' end of transcribed genes and using them to further high-throughput analysis such as sequencing.

【００１３】

【発明の実施の形態】

As described above, the method of the present invention can comprise but is not limited to roughly three steps each of which further comprises a plurality of steps. Each step will now be explained below. The concrete working examples of each step is described in detail in the later-mentioned working examples.

【００１４】

Step 1

Step 1 is a step to selectively collect nucleic acids said cDNAs containing a site corresponding to the 5' end site of mRNAs within a sample and which are synthesized for instance by using said mRNAs as templates.

【００１５】

Either total RNA or mRNA taken from a desired cell or tissue can be used as the starting substrate. The preparation method of total RNA and mRNA is already known, and it is also described in detail in the later-mentioned working examples. In other embodiments, a full-length cDNA librar

y may be used to isolate the 5' end nucleic acids corresponding to the 5' end of the transcribed part of the genes. Alternatively, a cDNA library itself would be cleaved if it carries a Class IIs enzyme in proximity of the 5' end.

【００１６】

Step 1 itself can be conducted by a publicly known method. In other words, methods to construct full-length cDNAs and methods to synthesize cDNA fragments at least containing a site corresponding to the 5' end site of the mRNAs are already known, and any of these methods can be adopted. One of the preferable methods is the cap trapper method (e.g. Piero CARNINCI et al., METHODS IN ENZYMOLOGY, VOL. 303, pp. 19-44, 1999). This cap trapper method shall be explained below, however, the invention is not limited to the use of the cap trapper method and other approaches to enrich or select full-length cDNAs could be applied as well. An alternative method (as described by Pelletier et al. in 1995) makes use of an immobilized cap-binding protein to isolate full-length cDNAs after RNase treatment of a hybrid.

【００１７】

Alternatively to the cap-selection, one could dephosphorylate with a phosphatase, such as BAP (bacterial alkaline phosphatase) the 5' ends of mRNAs, followed by treatment with the decapping enzyme TAP (tobacco acid pyrophosphatase). Subsequently a ribonucleotide or a deoxyrubonucleotide can be attached to the 5' end of the mRNA instead of the original cap-structure with RNA ligase (Maruyama and Sugano) In this way, for instance, a Class II S recognition site could be placed on the oligonucleotides/ribonucleotide sequence using during the ligation step, which is placed at the 5' end of a cDNA or RNA. This class II s restriction enzyme can then cleave the cDNA and produce the 5' end tag.

【００１８】

Alternatively to biotin, a cap-binding protein (Pelletier et al. Mol C ell Biol 1995 15:3363-71) or an antibody that specifically binds to the cap structure can be used as the aforementioned selectively binding subs tance.

【００１９】

Alternatively, one could use methods to attach oligonucleotides chemic ally to the cap structure as described by Genset. This method is based o n the oxidation of cap (US patent 6,022,715). This allows (1) adding to the cap an oligonucleotides, which may contain the ClassIIs enzyme, (2) preparing first-strand cDNA synthesis which then switched second strand cDNA synthesis; after the second strand synthesis, the cDNA would be cle aved with Class II s enzymes to make a 5' tag, for subsequent formation of the concatamer.

【００２０】

Alternatively, one could use the Use the cap-switch method as describe d by Clontech (US patent 5,962,272). One could prepare the first-strand cDNA in presence of a cap-switch oligonucleotide, which carries a recogn ition site for a substance capable to recognize nucleic acids and cleave them apart from the said recognition sequence such a Class II s restric tion enzyme site. The cap switch mechanism let the first strand sequence continue on the cap-switch oligonucleotides. This can be followed by se cond cDNA strand, possibly also followed by PCR (as describes for instan ce in the SMART™ Clontech cloning system), and finally it would be clea ved with the class II s to produce the 5' end TAGS.

【００２１】

In another embodiment, when the quality of RNA allows it, one can prep are the cDNA by priming and extending the RNA until the cap-structure. P articular enzyme and reaction condition allow sometimes reaching the cap -site very efficiently (Carninci et al, Biotechniques, 2002). Even witho

ut a cap-selection it is possible to attach oligonucleotides in place of the cap structure, which carry Class IIs restriction enzyme sites that would be later used to produce concatamers.

【００２２】

The cap trapper method first synthesizes the first-strand cDNA with a reverse transcriptase by using RNA as a template. This can be conducted by a known method. The cDNA can be primed with an oligo-dT primer or, when the template RNA is mRNA, it can be primed with a random primer. It is advisable to add trehalose to the reactive solution because it raises the efficiency of reverse transcription reaction by stabilizing the reverse transcriptase. It is preferable to use 5-methyl-dCTP instead of standard dCTP, because it avoids internal cDNA cleavage with several restriction enzymes and prevents unintended cleavage with restriction enzymes to a considerable extent. In addition, after the first-strand cDNA synthesis, proteins and digested peptides might be removed by CTAB (cetyl trimethyl ammonium bromide) treatment, or other more general methods to purify cDNA.

【００２３】

Next, a selectively binding substance is bound to the cap structure of mRNA. A "selectively binding substance" here means a substance that selectively binds to a specific substance, preferably but not limited to biotin. The cap structure is the structure at the 5' end of mRNA, which does not exist in transfer RNA (tRNA) or ribosomal RNA (rRNA). Therefore, even if total RNA was used as the starting substrate, the selectively binding substance only binds to mRNA. In addition, the selectively binding substance does not bind to mRNA if the cap structure at the 5' end has been cleaved. Biotin can be bound to the cap structure by a known method. For instance, the cap structure can be biotinylated by first oxidizing the diol groups on the cap structure by treating mRNA with an oxidizer

such as $NaIO_4$ and making then react with biotin hydrazide. Alternatively, any other methods known to a person trained in the state of the art of the preparation of full-length cDNAs can be utilized to selectively enrich 5'-ends according to the invention.

【００２４】

Then, single-strand RNA is cleaved by means such as RNase I treatment. Any other RNase that can cleave single strand RNA but not cDNA/RNA hybrids or cocktails of RNAses that can cleave the various single-strand RNAs sequences at various specificity can be used alternatively. In an RNA/cDNA hybrid whose first-strand cDNA has not extended to the site corresponding to the 5' end site of RNA, the vicinity of the 5' end of RNA is single-stranded due to its failure to be hybridized with cDNA. Thus, the hybrid is cleaved at the single-stranded part and loses its cap structure through this step. Consequently, this step leaves only those mRNA/cDNA hybrids with cDNA that fully extends to the 5' end of mRNA to maintain the cap structure.

【００２５】

A matching selectively binding substance fixed to a support, which selectively binds to the aforementioned selectively binding substance, is prepared. In the present specification, a "matching selectively binding substance" means a substance that selectively binds to the aforementioned selectively binding substance, which, in the case where the selectively binding substance is biotin, would be avidin, streptavidin or a derivative thereof that binds specifically to biotin or its derivatives. The support can favorably be, but is not limited to be, magnetic beads, particularly magnetic porous glass beads. Since magnetic porous glass beads to which streptavidin has been fixed are commercially available, such commercial streptavidin-fixed magnetic porous glass beads can be used favorably. Similarly other materials such as latex beads, latex magnetic bead

s, agarose beads, polystyrene beads, sepharose beads or alike could be u sed instead of porous glass beads. Furthermore, the invention is not lim ited to the use the biotion-avidin system but other binding substances c ould be used like a digoxygenin tag that would be attached to the cap st ructure and digoxygenin recognizing antibodies attached to a solid matri x .

【００２６】

Following this, the aforementioned mRNA/cDNA hybrid with the cap struc ture is made to react with the aforementioned matching selectively bindi ng substance fixed to the support in order to bind the selectively bindi ng substance on the cap structure with the matching selectively binding substance on the support, thereby immobilizing the mRNA/cDNA hybrid with the cap structure on the support. When magnetic beads are used as the s upport, the magnetic beads can be quickly collected by applying a magnet ic force. As mentioned above, the mRNA/cDNA hybrids that have the cap st ructure at this stage are only those with cDNA that fully extends to the 5' end of mRNA, so cDNAs containing a site complementary to the 5' end of mRNAs are selectively collected by this step, and Step 1 is completed . Meanwhile, in order to prevent non-specific binding to the support, it is preferable to treat the support with a large excess of DNA-free tRNA for blocking such binding before conducting this reaction. Other substa nces that are suitable for blocking the surface are nucleic acids or der ivatives, for instance total RNA or oligonucleotides; proteins, for inst ance bovine serum albumine; polysaccharides, for instance glycogen, dext ran sulphate, heparin or other polysaccharides. Alternatively, hybrid mo lecules containing parts of all of the above could be used to mask non-s pecific binding sites.

【００２７】

The above focuses on the case where Step 1 is conducted by the cap tra

pper method, but other various methods can also be used as indicated as long as they can selectively collect cDNAs containing a site complementa ry to the 5' end site of mRNA.

【００２８】

The following Step 2 selectively collects fragments containing a cDNA site that at least contains a site complementary to the 5' end site of m RNA.

【００２９】

First, the first-strand cDNA that has been immobilized on the support is released. It can be conducted by treating the support with alkali, su ch as NaOH. Alternatively to alkali, an enzymatic reaction with RNaseH (which cleaves only the RNA hybridized to DNA) could be used. The alkali treatment releases the cDNA from the mRNA/cDNA hybrid, bound to the supp ort through the cap on the mRNA and separates the cDNA from the mRNA to only leave first-strand cDNA on its own.

【００３０】

Then, a linker carrying a sequence that can be recognized in a sequenc e-specific manner by a substance having an enzymatic activity that cleav es the recognized DNA outside the recognition sequence. An example of su ch substance is a Class IIs restriction enzyme.

【００３１】

In this embodiment, a linker that at least carries a Class IIs restric tion enzyme site, and a random oligomer part at the 3' end site, is liga ted to the end of this first-strand cDNA, which corresponds to the 5' en d of the aforementioned mRNA (i.e. the 3' end of the cDNA). For the late r cloning of the 5' end sequence tags into concatemeres it is preferable but not essential to introduce a second recognition site into the linke r, which should be distinct from the aforementioned recognition site use d for the e.g. Class IIs restriction enzyme.

【００３２】

This can preferably be conducted as follows, by a method using a linker that carries a Class IIs restriction enzyme site and a random oligomer part (SSLLM (single strand linker ligation method), Y. Shibata et al., BioTechniques, Vol. 30, No. 6, pp. 1250-1254, (2001)). The Class IIs restriction enzyme is a restriction enzyme group that causes cleavage at parts other than the recognition site. An example includes but is not limited to the use of GsuI. GsuI treatment cleaves one of the strands at 16 bp downstream from the recognition site, and the other strand at 14 bp downstream from the recognition site. Another suitable example is MmeI, which cleaves respectively 20 and 18 bases apart its recognition sequence. The random oligomer part is located at the 3' end site of the linker, and though the number of bases is not particularly restricted, the recommended number is 5 to 9, or more preferably, 5 to 6. The Class IIs restriction enzyme site should be located close to the aforementioned random oligomer part, so that the cleavage point comes within the cDNA, particularly relatively within the 5' side of the cDNA (i.e. the 3' side of the template mRNA). The linker should preferably be a linker for double-stranded DNA of which the aforementioned random oligomer part protrudes to the 3' side and provides the binding end. In addition, it is advisable to bind a selectively binding substance such as biotin to the linker in advance to facilitate its collection later.

【００３３】

When the aforementioned first-strand cDNA is made to react with such a linker, the random oligomer part of the linker hybridizes with the 3' end site of the first-strand cDNA (i.e. the 5' end site of the template mRNA). Next, the second-strand cDNA is synthesized by using this linker as a primer and the first-strand cDNA as a template. This step can be con

ducted by a standard method.

【００３４】

Then, the obtained double-strand cDNA is treated with the above Class IIs restriction enzyme. This step produces a double-strand cDNA fragment comprising a linker-derived part and a part derived from the 5' end site of the cDNA (the 5' end site of the second-strand cDNA). For instance, if GsuI were to be used as the Class IIs restriction enzyme and if there were to be a linker designed to locate the restriction site immediately upstream from the aforementioned random oligomer site, the obtained DNA fragment would include a site derived from the site on the 5' end side of the second-strand DNA (i.e. the site on the 5' end side of the mRNA) of the length of 16 bp (however, the complementary strand is 14 bp). In the case of the use of Mme I the length of the second-strand DNA fragment should increase to 20 and 18 bp respectively.

【００３５】

Next, such DNA fragments are selectively collected. If a selectively binding substance (e.g. biotin) had been bound to the linker as above, the collection could be conducted similarly to Step 1 by using a support to which a matching selectively binding substance (e.g. streptavidin) would be fixed. This procedure completes Step 2, which selectively collects fragments containing a cDNA site, belonging to the first-strand cDNA, which at least contains a site complementary to the 5' end site of the aforementioned mRNA.

【００３６】

The above explains the case where the SSLLM is used for Step 2, but Step 2 can also be carried out by any other method as long as the method can selectively collect fragments containing the 3' end site of the first-strand cDNA (the 5' end site of the template mRNA). For instance, it is possible to use exonuclease that cleaves the nucleotide in the 5'-3' di

rection at a controlled speed. The exonuclease treatment of the first-st rand cDNA for a prescribed time period leaves a single-strand fragment c omprising the 3' end site of the first-strand cDNA (the 5' end site of t he template mRNA). It is possible to obtain only the targeted single-str and fragments by conducting treatment with a nuclease that only splits d ouble-strand fragments. These fragments can be collected, joined with ad apters and cloned.

【００３７】

The subsequent Step 3 forms concatamers by mutually ligating the colle cted fragments. Since there are multiple mRNAs and the linker hybridizes with the first-strand cDNA at the random oligomer part as above, the ab ove method can obtain fragments containing multiple cDNAs derived from m ultiple mRNAs within a sample. Step 3 litigates these multiple fragments and forms concatamers. The ligation of the cDNA fragments can be carrie d out by a standard method, using commercial ligation kits. The ligation can be securely conducted but is not limited to a method which first is introducing a second linker providing a recognition site for a restrict ion enzyme that is distinct form the other recognition sites used at the earlier stages, which is then ligating two fragments into di-tags, and which is further ligating such ligated di-tag fragments into concatamers . The number of ligated fragments is not restricted, practically any num ber above two and preferably about 30. The obtained concatamers are pref erably but not limited to be amplified or cloned by a standard method.

【００３８】

The concatamers obtained in this way each comprise a site having the s ame base sequence (however, uracil in RNA would be thymine in DNA) as th at of the 5' end site of the multiple mRNAs within the sample. Although it also comprises a part derived from the linker or linkers, the base se quence of the linker or linkers is already known, so the part derived fr

om the linker or linkers and the part derived from mRNA can be clearly d istinguished by investigating the base sequence of the concatamer. There fore, by determining the base sequence of the obtained concatamer, it is possible to find out the base sequences at the 5' end site of multiple mRNAs within the sample. The base sequences of a maximum of 16 or 20 bas es at the 5' end site of each mRNA can be learned by the preferable mode of using GsuI or Mme I. Information on 16 or 20 bases would be sufficie nt for almost definitely identifying the mRNA statistically and to judge whether or not it is a new mRNA. In addition, by determining the base s equence of the concatamer, it is possible to learn the base sequences at the 5' end site of mRNAs for the number of above fragments included in the concatamer (preferably 20 to 30), so information on the 5' end site of multiple mRNAs can be determined efficiently. The analysis of the con catamers can be automated by the use of computer software to distinguish between sequences derived form the 5'-ends and sequences derived form a linker or the linkers.

【００３９】

When a new mRNA exists in a base sequence at the 5' end, the cDNA deri ved from the new mRNA can be obtained by conducting RT-PCR, making that site the forward primer and oligo-dT the reverse primer. It is also poss ible to amplify the mRNA by methods such as NASBA. Accordingly, the meth od of the present invention can be used for the cloning of new genes. Si milarly, forward primers derived form 5'-end specific information can be used to amplify partial or full-length cDNA fragments from exciting cDN A libraries.

【００４０】

While the above method had used mRNA or total RNA within the sample as the starting substrate, Step 1 can be omitted by using an existing full -length cDNA library. In this way, information on the base sequences of

the 5' end site of multiple cDNAs (i.e. the 5' end site of the mRNAs used as templates for said cDNAs) contained in the full-length cDNA library can be efficiently obtained similarly to the above procedure.

【００４１】

In some embodiments it could be desirable to obtain extended sequence information from the 5'-ends of transcribed regions. Such extended sequences may allow in specific cases for the identification of start sites of protein synthesis or a better mapping to genomic sequences. As described above the invention included in Step 2 the ligation of a linker to the 5' end of a cDNA. Such a linker can be modified by introducing a single-stranded overhang encompassing a sequence obtained from a concatamer to bind to and to be ligation to a specific nucleic acid fragment. After the ligation the linker can be used to enrich the DNA fragment by attaching the linker to a support from which it could be released after the enrichment. The linker can further be used as a primer to obtain extended sequence information on 5' ends.

【００４２】

By investigating the base sequences of the concatamers or extended 5'-sequences obtained by the present invention, it is not only possible to clone new genes as described above, but also possible to investigate the expression profiles of genes within the sample. Furthermore, the technology can be used for various purposes such as to map transcription start sites in the genome, to map promoter usage patterns, for the analysis of SNPs in promoter regions, for creating gene networks by combining the expression analysis with information on promoters, alternative promoter usage and the other data, and for selective collection of the promoter site within fragmented genomic DNA. To select genomic fragments containing promoter sites, a fragment containing the same base sequence as the 5' end site of mRNA could be bounded to a support e.g. by using the aforem

entioned Biotin system, and hybridized to fragmented genomic DNA. Hybridized genomic DNA fragments could then be separated from a mixture of genomic fragments by using e.g. streptavidin-fixed magnetic beads, and cloned under standard conditions.

【００４３】

Alternatively, one could avoid to make concatamers and use selected 5' end tags by ligating a mixture of full-length cDNAs to magnetic beads carrying homogeneous sequence of oligonucleotides, followed by ligation such as in the SSLLM, second strand cDNA preparation and cleavage with a Class IIs restriction enzyme. The 5' end specific tag would be anchored specifically to the beads and would be used for the specific sequencing as done by Lynx therapeutics (US patents 6,352,828; 6,306,597; 6,280,935; 6,265,163; 5,695,934).

【００４４】

For instance, oligonucleotides would have a "random part I", which will bind to 5' ends of cDNAs; and a code part of the oligonucleotide, which will be able to "tag" the ligation product. The oligonucleotide may be destroyed by exonuclease VII if not hybridized with a cDNA. The "decoder" oligonucleotides would be used to select out the sequence. The specific arrays of cDNAs on beads are then arrayed onto a solid surface, one per position, followed by parallel sequencing. If you look at 1 hole per 1 bead, you can make arrays of beads having specific oligonucleotides.

【００４５】

By modifications as the aforementioned approaches for direct sequencing of 5' end the invention provides different means for the general analysis of 5' ends in the form of concatamers or the analysis of individual 5' ends, which were enriched by means of a 5' end specific selection.

【００４６】

【実施例】

The present invention will now be described by way of examples thereof. It should be noted that the present invention is not restricted to the Examples. The experiments describe in the Examples can be performed by any person experienced in the state of the art of standard techniques in the field of Molecular Biology. Unless otherwise defined in the text, the technical terms, abbreviations, and solutions used in the Examples should have the same meaning as commonly understood by a person experienced to the state of the art in the field of the invention. A general description of such terms, abbreviations and solutions can be found in the common reagent section in Molecular Cloning (Sambrook and Russel, 2001). All publications mentioned herein are incorporated into this document by reference to be disclosed and to describe the methods and/or materials therein.

【００４７】

Example 1:

Preparation of total RNA from tissue

In the literature a variety of different approaches for the preparation of RNA have been described, which are known to a person experienced in the state of the art. All such approaches should allow the preparation of a plurality of RNA samples derived from biological materials including tissues and cells, which are suitable for the invention. Below two such procedures are described in detail.

Buffers and solutions:

a) Solution D: 4M guanidinium thyocyanate, 25mM sodium citrate (pH7.0), 100mM 2-mercaptoethanol and 0.5% n-lauryl-sarcosine.

b) RNase-free CTAB/UREA solution: 1% CTAB (Sigma), 4M UREA, 50mM Tris-HCl (PH 7.0), 1mM EDTA (pH 8.0).

c) Water equilibrated phenol as described in Molecular Cloni

ng (Sambrook and Russel, 2001).

Phosphate-buffer saline (PBS) as described in Molecular Cloning (Sambrook and Russel, 2001)

5 M Sodium chloride

7 M Guanidium choride

Rnase free dd-water

【００４８】

Protocol for total RNA preparation

Dissect the tissue as fast as possible in a cooled dish.

Roughly evaluate the volume of tissue in a 50 ml falcon tube. The best quantity of tissue is between 0.5-1 g of tissue for 20 ml Solution D

Add 2 ml of 2M sodium acetate (pH 4.0) and 16 ml of water-equilibrated phenol.

Mix by a vortex. Add 4 ml of chloroform and shake vigorously by your hands and a vortex. Let it stay on ice for 15 min.

Centrifuge it at 6,000 rpm for 30 min at 4 °C

Transfer the upper aqueous phase to new tube by pipetting (25 ml) and recover approximately 20 ml thereof.

Precipitate the RNA from the aqueous phase by adding 1 equal volume of Isopropanol (in this case, approximately 20 ml), store on ice for 1 h.

Centrifuge at 7,500 rpm for 15 min at 4 °C: RNA is pelleted by centrifugation.

The pellet is washed twice with 70% ethanol, each time followed by centrifugation at 7,500 rpm for 2 min, in order to remove the SCN salts.

CTAB removal of polysaccharides. Selective CTAB precipitation of mRNA is performed after complete RNA re-suspension in 4 ml of water. Subsequently, 1.3 ml of 5 M NaCl is added and the RNA is then selectively precipitated by adding 16 ml of a CTAB/urea solution.

Centrifuge for 15 min at 7500 rpm (9500 x g), discard the aqueous phase.

Resuspend the RNA pellet in 4 ml of 7 M Gunidinum Cloride.
Re-suspended RNA is finally precipitated by adding 8 ml of ethanol. Incu bate on -20ºC for 1-2 hours (or longer) and centrifuge for 15 min at 7,5 00rpm, 4℃. At the end, wash the pellet with 5 ml of 70% ethanol.
Centrifuge again at 7,500 rpm for 5 min.
Discard the supernatant.
Re-suspend RNA in 500-1000 microL of RNase-free dd-water.

【００４９】

Preparation of a mRNA fraction from total RNA

The mRNA fragtion of total RNA preparations can be isolated by the use of commercial kits such as the MACS mRNA isolation kit (Milteny) or pol yA-quick (Stratagene), which provide satisfactory yield of mRNA under th e recommended conditions. One cycle of oligo-dT selection of the mRNA is sufficient. It is advisable to redissolve the poly-A^+ RNA at a high con centration of 1 to 2 microG/microL.

【００５０】

Preparation of a plurality of RNA samples from a cDNA library

Alternatively, a plurality of nucleic acids corresponding to the 5' en ds of genes can be obtained from existing cDNA libraries, which were clo ned into expression vectors. By standard methods known to a person famil iar with the state of the art of molecular biology approaches, from such libraries RNA transcripts can be obtained by in vitro transcription rea ctions using e.g. a T3, T7 or SP6 RNA polymerase. Such an approach can b e performed by first linearization of the plasmid DNA with appropriate r estriction endonucleases. The restriction enzyme can be chosen to allow for the transcription of the sense RNA. In the case of libraries obtaine d in the vector pFLC III (Carninci P, Shibata Y, Hayatsu N, Itoh M, Shir aki T, Hirozane T, Watahiki A, Shibata K, Konno H, Muramatsu M, Hayashiz aki Y.) Balanced-size and long-size cloning of full-length, cap-trapped

cDNAs into vectors of the novel lambda-FLC family allows enhanced gene discovery rate and functional analysis, Genomics, 2001 Sep;77(1-2):79-90), the vector can be linearized by cleavage with one of the homing endonucleases I-Ceu I or PI-Sce I to avoided a truncation of the inserts. For the digest mix in a tube

Plasmid DNA 100 microG

10x buffer 40 microL

Restriction enzyme 100 u

ddH_2O ad 400 microL

Incubate at appropriate temperature for at least 2h and analyze 1 microL of the reaction mixture by agarose gel electrophoreses. If the digest is completed, add:

0.5 M EDTA 8 microL

10% SDS 8 microL

Proteinase K (10 mg/ml) 5 microL

Incubate for 15 min at 45°C before extracting sample with 500 microL phenol/chloroform. The aqueous phase is to be re-extracted twice with 500 microL chloroform. Finally linearized DNA is precipitated with isopropanol or ethanol under standard conditions and dissolved in 50 microL TE.

【００５１】

In vitro RNA synthesis:

Mix in a tube under Rnase free conditions:

Linearized plasmid DNA 20 microG

5x T7 or T3 buffer 200 microL

0.1 M DTT 100 microL

2 mg/ml BSA 40 microL

10 mM rNTPs 50 microL

T7 or T3 RNA polymerase 10 microL

ddH_2O ad 1000 microL

Incubate at 37°C for 3 to 4 h before adding:

10 mM Calcium Chloride 10 microL

1U/microL DNase RQ1 5 microL

Incubate at 37°C for 20 min before adding:

0.5 M EDTA 10 microL

10 mg/ml Protease K 5 microL

Incubate at 45°C for 30 min, before addition of Sodium Chlorid to a final concentration of 1M. Phenol/Chloroform extraction followed be re-extraction with Chloroform should be performed under standard conditions, and the RNA transcripts can be finaly collected by Isopropanol or Ethanol precipitation. The pellet is to be resuspended in 200 microL of water or TE. The quality of the RNA transcripts should be confirmed by agarose gel electrophorese and quantification.

【００５２】

2. : First strand cDNA synthesis

Buffers and solutions

Saturated Trehalose, about 80% in water (crystals will remain), low metal content

4.9 M high purity sorbitol

Optionally: Takara GC-Taq buffer

【００５３】

Enzymes and buffers

RNase H- reverse transcriptase Superscript II (Invitrogen) and buffer or other reverse transcriptases.

【００５４】

Nucleic acids and oligonucleotides

Purified, first-strand oligo-dT primer (Sequence for primer used: 5'-GAGAGAGAGAGGATCCTTCTGGAGAGTTTTTTTTTTTTTTTTVN-3'). Alternatively or additionally, random primer (dN_6-dN_9), where N is any nucleotide.

mRNA, recommended 2.5 to 25 microG or alternatively, total RNA, 5-50 microG

【００５５】

Radioactive compounds

[alpha-^{32}P] dGTP

【００５６】

Protocol A: Trehalose-Sorbitol enhanced

To prepare the 1st strand cDNA, put together the following reagents in three different 0.5 ml PCR tubes (A, B, and C)

【００５７】

Tube A: in a final volume of 21.3 microL, add the following:

mRNA 2.5-25 microG

or total RNA, 5-50 microG

1st strand primer (2 microG/microL) 14 microG (7 microL)

Total volume: 22 microL

Heat the mixture (mRNA, primer) at 65°C for 10 min to dissolve the secondary structures of mRNA.

Tube B: in a final volume of 76 microL, add the following:

5X 1st strand buffer 28.6 microL

0.1 M DTT 11 microL

dATP, dTTP, dGTP, and 5-methyl-dCTP 10 mM each 9.3 microL

4.9 M sorbitol 55.4 microL

Saturated trehalose 23.2 microL

RNase H- Superscript II reverse transcriptase (200 U/microL)

15.0 microL

Final volume: 142.5 microL

【００５８】

Prepare a cycle (on a thermal cycle) with: 40°C, 4 min; 50°C, 2 min; 56°C, 60 min.

If total RNA is used as the starting material, prepare a cycle with:
40°C, 2 min, -0.1°C/sec to 35°C; 50°C, 2 min; 56°C, 60 min.
Alternatively: prime the cDNA with a random primer (dN_9, N= any nucleotide) at 25° C.

【００５９】

Tube C:

1~1.5 microL of [alpha-^{32}P] dGTP.

【００６０】

For a cold-start operate as follows:
Quickly mix tubes A and B on ice.
Transfer in tube C 40 microL of the A+B mixture.
Tubes A+B and C should be quickly transferred immediately at 40°C of the step 1 of the above cycling program to anneal at 40°C four 4 minutes.
Let the reaction proceed following the thermal cycler setting.

【００６１】

For a hot-start, operate as follows:
Transfer the tubes A, B, C on the thermal cycler
Start the cycling
When the temperature reaches 42°C, quickly mix tubes A and B.
Transfer in tube C 40 microL of the A+B mixture.
Let the reaction proceed following the thermal cycler setting.

【００６２】

Protocol B: GCI-Trehalose-Sorbitol enhanced
Tube A: in a final volume of 22 microL, add the following:
mRNA　　5-25 microG
(precipitate with ethanol and re-suspend directly with the primer)
or total RNA, up to 50 microG (for the small-scale protocol)
Purified 1st strand cDNA primer (2 microG/microL)14 microG(7 microL)
Final volume: 22 microL

Tube B: add the following:

2 X GC I (LA Taq) buffer (TaKaRa) 75microL

dATP, dTTP, dGTP, and 5-methyl-dCTP, 10 mM each 4 microL

4.9 M sorbitol 20 microL

Saturated trehalose (approximately 80%) 10 microL

Superscript II reverse transcriptase (200 U/microL) 15 microL

ddH_2O 4 microL

Final volume: 128 microL

Tube C:

alpha-^{32}P-dGTP 1.5 microL

For the rest of the procedure, follow exactly the point as in the normal reaction condition. Prepare (in advance) a thermal cycler with the following cycle:

42°C, 30 min; 50°C, 10 min; 55°C, 10 min; 4°C, indefinite time.

【００６３】

Operate as follows:

1) Transfer the tubes A, B, C on the thermal cycler

2) Start the cycling

3) When the temperature reaches 42°C, quickly mix tubes A and B.

4) Transfer in tube C 40 microL of the A+B mixture.

5) Let the reaction proceed following the thermal cycler setting.

At the end, stop the reaction with EDTA at 10 mM final concentration.

Then incorporation of [alpha^{32}P]GTP is measured and the yield of cDNA is calculated. Calculation of the amount of cDNA by measuring [alpha^{32}P]GTP is useful for monitoring whether the processes are accurately proceeding or not.

【００６４】

3. CTAB precipitation of the first-strand cDNA

Buffers and solutions

CTAB solution as described in Example 1

After measuring the radioactivity, transfer both the "hot" and "cold" 1st strand synthesis (tube B and C) to a tube and perform CTAB precipitation as follows.

Mix the tube B and C from the first strand; to the mixture add:

3 microL of 0.5 M EDTA (final concentration of 10 mM)

2 microL of 10 microG/microL Proteinase K.

Incubate at 45°C or 50°C for at least 15 min, and as long as 1 hour.

To the 128-142 microL volume of the first strand cDNA reaction, add:

32 microL of 5 M Sodium Chloride (RNase free)

320 microL of CTAB-Urea solution

Incubate at room temperature for 10 min.

Centrifuge at 15,000 rpm for 10 min

Remove supernatant.

Carefully re-suspend with 100 microL of 7M Guanidinium Cloride

Add 250 microL of ethanol and leave on ice or 20 to -80°C for 30-60 min

Centrifuge at 15,000 for 10 min. Remove the supernatant.

Subsequently, wash the pellet twice with 800 microL of 80% ethanol. Each time, add 80% ethanol to the tube and centrifuge for 3 min. at 15,000 rpm.

Re-suspend cDNA in water 46 microL.

【００６５】

4. Cap-trapping, oxidation and biotinylation of the cap

Buffers and solutions

1 M sodium acetate buffer, pH 4.5

1M citrate buffer, pH 6.0

$NaIO_4$, solution >100 mM.

SDS 10%

Biotinylation buffer: 33 mM Sodium citrate, pH 6.0, and 0.33% SDS.

10 mM Biotin Hydrazide long arm (MW = 371.51; 3.71 mg/ml = 10 mM) in citrate/SDS buffer.

Cap biotinylation: (A) Oxidation of the diol groups of mRNA

In a final volume of 50 to 55 microL, add the following:

The re-suspended cDNA sample

3.3 microL of 1 M sodium acetate buffer, pH 4.5

A freshly prepared solution of $NaIO_4$ to a final concentration of 10 mM

Incubate on ice in the dark for 45 min.

Finally, precipitate the cDNA:

To simplify the downstream process, add 1 microL of glycerol 80%.

Vortex.

Add 0.5 microL of 10% SDS, 11 microL of 5 M sodium chloride and 61 micro L of isopropanol.

Incubate at 20 or -80°C for 30 min in the dark.

Centrifuge for 15 min at 15,000 rpm.

Remove supernatant.

Add 500 microL of 80% ethanol

Centrifuge at 15,000 rpm for 2-3 min.

Discard the supernatant

Repeat steps 12-13

Re-suspend the cDNA in 50 microL of water.

Biotinylation: (B) Derivatization of the oxidized diol groups

To the cDNA (50 microL), add 160 microL of the dissolved biotin hydrazid e long arm in the reaction buffer. Perform the reaction in 210 microL (f inal volume).

Incubate overnight (10-16 hours) at room temperature (22-26°C).
Subsequently, to precipitate the biotinylated cDNA, add:
75 microL 1 M Sodium citrate, pH 6.1
5 microL of 5 M Sodium chloride
750 microL of absolute ethanol
Incubate on ice for 1 hour or at 80 or -20°C for 30 min or longer.
Centrifuge the sample at 15,000 rpm for 10 min
Wash the precipitate twice with 70% or 80% ethanol and centrifuge.
Discard the supernatant and repeat the wash. Dissolve the cDNA in 175 microL of TE (1 mM Tris, pH 7.5, 0.1 mM EDTA).
Cap-trapping and releasing the 5' ends of cDNA Enzymes and buffers
RNase ONE (Promega) and its reaction buffer

To the cDNA sample add, in a final volume of 200 microL:
20 microL of RNase I buffer (Promega).
1 units of RNase I (Promega, 5 or 10 U/microL) per each 1 microG of starting mRNA or total RNA (in case of small scale protocol) used for first strand cDNA synthesis.
Incubate at 37°C for 30 min.
To stop the reaction, put the sample on ice and add
4 microL 10% SDS and
3 microL of 10 microG/microL Proteinase K.
Incubate at 45°C for 15 min.
Extract once with 1:1 Tris-equilibrated phenol:chloroform, then load the aqueous phase into Microcon -100.
Perform a back extraction with water and load again into the Microcon-Centricon 100 filter.
Perform one round of Microcon separation
8-b) Dissolve completely the pellet with 20 microL of 0.1 x TE

【００６６】

Magnetic beads blocking

Materials

Streptavidin-coated MPG (CPG inc., New Jersey)

Buffers and solutions

Binding buffer: 4.5 M NaCl, 50 mM EDTA, pH 8.0

Special equipments

A magnetic stand to hold 1.5 ml tubes is required.

To further minimize the non-specific binding of nucleic acids, magnetic beads are pre-incubated with DNA-free tRNA (10mg/ml).

For each preparation, pre-incubate 500 microL of magnetic beads (per 25 microG of starting mRNA) with 100 microG of tRNA.

Incubate on ice for 30 min with occasional mixing.

Separate the beads with a magnetic stand (for 3 min) and remove the supernatant.

Wash for 3 times with 500 microL of binding buffer

【００６７】

5'-ends cDNA capture and release

To capture the full-length cDNA, mix the RNaseI-treated cDNA and wash beads as follows:

1) Re-suspend the beads in 500 microL of wash/binding buffer.

2) Transfer 350 microL of the beads into the tube containing the biotinylated first-strand cDNA.

3) After mixing gently rotate the tube for 10 min at 50

°C,

4) Transfer 150 microL of the beads into the tube containing the biotinylated first-strand cDNA and 350 microL of beads.

5) After mixing gently rotate the tube for 20 min at 50 °C.

Separate the beads from the supernatant on a magnetic stand.

Washing the beads

Gently wash the beads with 0.5 ml of the indicated buffer to remove the nonspecifically absorbed cDNAs.

2 x with washing/binding solution.

1 x with 0.3 M NaCl/ 1mM EDTA

2 x with 0.4% SDS/ 0.5 M NaOAc/ 20 mM Tris-HCl pH 8.5/ 1mM EDTA.

2 x with 0.5 M NaOAc/ 10 mM Tris-HCl pH 8.5/ 1mM EDTA.

Alkali release (see below)

Alkali full-length cDNA release from beads

Add 100 microL of 50 mM NaOH, 5 mM EDTA.

Briefly stir and incubate 5 min at RT with occasional mixing.

Separate the magnetic beads and transfer the eluted cDNA on ice.

Repeat the elution cycle with 100 microL of 50 mM NaOH, 5 mM EDTA, two more times until most of the cDNA, 80-90% as measured by monitoring the radioactivity, can be recovered from the beads.

Adding a 5'-end primable site to the cDNA

RNase step

Enzymes and buffers

- RNase ONE™ and its buffer (Promega)

Add 50 microL of 1 M Tris-HCl, pH 7.0 in tubes on ice and mix quickly.

Add 1 microL of RNase I (10U/microL) and mix quickly.

Incubate at 37 °C for 10 min.

To remove the RNaseI, treat the cDNA with Proteinase K and phenol/chloro

form extraction including back extraction.

Add 3 microG of glycogen. Treat the cDNA with one cycle of Microcon-100.

Fractionation of cDNA before adding a primable site

Materials

Amersham-Pharmacia S-400 spun kit or alternative kits

Buffers and solutions

Column buffer: 10 mM Tris, pH 8.0, 1 mM EDTA, 0.1 % SDS, and 100 mM NaCl

Column buffer without SDS: 10 mM Tris, pH 8.0, 1 mM EDTA and 100 mM NaCl

S-400 spun column chromatography

Detailed protocols are described in the kits. This is the running protoc ol of S-400 spun columns.

Shake the column

Brake the seal and transfer in a 2 ml tube

Centrifuge at 3,000 rpm 1 min (+ 4 ^{o}C)

Add the cDNA (< 20 microL volume)

After cDNA, add 80 microL of water

Centrifuge 2 min at 3000 rpm

Concentrate by Microcon 100 or precipitate with isopropanol. Recovery s hould exceed 80%.

【００６８】

6. SSLLM

Materials

S-300 spun column chromatography kit (Amersham-Pharmacia)

Buffers and solutions

Column buffer: 10mM TrisHCl pH 8.0, 1mM EDTA, 0.1% SDS, 100mM NaCl.

Enzymes and buffers

Takara DNA Ligase KIT II.

Nucleic acids and oligonucleotides

In the Example given here, the recognition sites for the restriction enzymes Bgl II, Gsu I and Mme I are introduced, however, the invention is not dependent or limited to the use of those restriction enzymes and their recognition sites. In particular, Bgl II (recognition site: AGATCT) can be replaced by any endonuclease suitable for cloning. Other example for such enzyme could include Asc I (recognition site: GGCGCGCC) or Xba I (recognition site: TCTAGA).

- Synthesize the following oligonucleotides containing the GsuI restriction site.

Oligonucleotide Bg-Gsu-GN5:

5' -Biotin-AGAGAGAGAACTAGGCTTAATAGGTGACTAGATCTGGAGGNNNNN-3' ;

Oligonucleotide Bg-Gsu-N6:

5' -Biotin-AGAGAGAGAACTAGGCTTAATAGGTGACTAGATCTGGAGNNNNNN-3' ;

Oligonucleotide Bg-Gsu-down:

5' P-CTGGAGATCTAGTCACCTATTAAGCCTAGTTCTCTCTCT-NH_2 3'.

- Synthesize the following oligonucleotides containing the Mme I restriction site.

Oligonucleotide Bg-Mme-GN5:

5' -Biotin-AGAGAGAGAACTAGGCTTAATAGGTGACTAGATCTTCCRACGNNNNN-3' ;

Oligonucleotide Bg-Mme-N6:

5' -Biotin-AGAGAGAGAACTAGGCTTAATAGGTGACTAGATCTTCCRACNNNNNN-3' ; Oligonucleotide Bg-Mme-down:

5' P-GTYGGAGATCTAGTCACCTATTAAGCCTAGTTCTCTCTCT-NH_2 3' .

Where R stands for G or A and Y stands for C or T.

P means that the oligonucleotide must be 5' phosphorylated and NH_2 indicates that an amino-group is added to avoid non-specific ligation and possible hairpin priming.

Oligonucleotides should be purified by acrylamide gel electrophoresis following standard techniques as the first-strand cDNA primer with 10% acr

ylamide electrophoresis (Sambrook and Russel, 2001). Oligonulceotides sh ould be extracted with phenol/chloroform, chloroform and precipitation w ith 2 volumes of ethanol as for the first-strand cDNA primer.

【００６９】

Preparation of the linkers.

After OD checking and mixing Bg-Gsu-GN5, Bg-Gsu-N6 and "down" oligonucle otides at ratio 4:1:5, at least 2 microG/microL of DNA; add NaCl at 100 mM final concentration. The oligonulceotides are annealed at 65°C for 5m in, 45°C for 5min, 37°C for 10min, 25°C for 10min.

【００７０】

Ligation of the first-strand cDNA

Use 2 microG of linker mixture for up to 1 microG single-strand cDNA. Mi x linkers and cDNA (final volume: 5 microL)

Heat at 65°C for 5min to melt secondary structures of single-strand cDNA

Transfer the linker and cDNA mix on ice.

Add 5 microL of the solution II from the TAKARA DNA ligation Kit.

Add 10 microL of solution I of the kit.

Incubate at 10°C overnight (at least >10 hours).

At the end of the ligation reaction, stop the reaction by adding 1microL of 0.5 M EDTA, 1 microL of 10% SDS, 1microL of 10 mg/ml Proteinase K, 1 0 microL of water, and incubate at 45°C for 15 min.

Treat with phenol/chloroform, chloroform and back extract (see appendix) with 60 microL of column buffer

After the ligation, remove the excess linker with S-300 spin column chro matography

1) Shake the column several times and then let it stand upright.

2) Remove the upper cap, then the bottom one.

3) Drain the buffer of the column. Apply 2 ml of the column buffer and d rain twice by gravity.

Put the column into a 15 ml centrifuge tube, then centrifuge at 400 x g for 2 min in a swing-out rotor at room temperature.

Apply 100 microL of buffer to the column, then centrifuge at 400 x g for 2 min. Check the eluted volume. If it is different from the input (100 microL), repeat this step until the eluted volume is the same as the add ed one.

Set a 1.5 ml tube, after cutting off the cap, into the 15 ml centrifuge tube, and then apply the sample into the column. Centrifuge at 400 x g f or 2 min.

Collect the eluted fraction in a separate tube. Apply to the column 50mi croL of buffer, repeat the centrifugation and collect the fraction in a separate tube.

Repeat step 6 for 3 to 5 more times; keep the eluted fractions separate.

Collected fractions should be counted in a scintillation counter. Usuall y mix the first 2-3 fractions (80% of cpm of cDNA).

Add NaCl to a final concentration of 0.2 M, precipitated the cDNA by add ing equivalent of isopropanol.

After precipitation and washing twice with 80% cold ethanol, re-suspend with water.

Second-strand cDNA

Setting the 2nd strand cDNA program on the thermal cycler as follows:

Step 1	5 min at 65 ^{o}C
Step 2	30 min at 68 ^{o}C
Step 3	72 ^{o}C for 10 min
Step 4	+4^{o}C

Procedure for the second strand cDNA

Second strand steps, mix in a test tube:

The cDNA

6 microL of LA-Taq polymerase buffer (Takara)

6 microL of 2.5 mM (each) dNTP's (Takara)

0.5 microL of [alpha-^{32}P] dGTP (optional to follow the incorporation)

After starting the 2nd strand program, put the tube on the thermal cycle r.

Add to tube 3 microL of 5 U/microL of LA Polymerase or alternative therm ostabe polymerase cocktails, when the samples are at 65°C, during the fi rst step.

Mix quickly but thoroughly

At the end of the cycle of the thermal cycler, stop the reaction by addy ing 10 mM EDTA (final concentration) and clean up the reaction by Protei nase K treatment, Phenol-chloroform extraction and ethanol precipitation (see Sambrook and Russel, 2001, Molecular Cloning, CSHL press, NY).

【００７１】

11.　　Cleavage of cDNA

The cDNA should then be cleaved with the Class IIs restriction enzyme li ke Gsu I given in this Example.

Buffer (10X) (MBI Fermentas)	10 microL
GsuI(1U/microL) (use 5U/microG DNA)	Y microL
ddH_2O	X microL
Final volume	100 microL

Where the Y and X vary depending on the quantity of cDNA

1)　　Incubate at 37℃ for 1 hour.

2)　　Added 0.5M EDTA 2 microL.

3)　　Incubated at 65℃ for 15 min. to inactivate the enzyme

Prepare the magnetic beads

Prepare the appropriate quantity of CPG-MPG (Magnetic porous glass beads

). The same considerations made for the cap-trapper step are valid at th is point.

Prepare 200 microL of GPG- beads.

Add 5 microG of tRNA (20 mg/ml).

Incubate at RT for 10-20 min or on ice for 30-60 min, with occasional sh aking

Transfer the beads on a magnetic stand for 3 minutes and remove the aque ous phase.

Wash 3 times with: 1M NaCl, 10 mM EDTA use at least a volume equivalent to the starting volume of beads.

Re-suspend beads in 1M NaCl, 10 mM EDTA equivalent to the starting volum e of beads.

【００７２】

7. Released cDNA tags

Mixed washed beads and GsuI cut sample.

Incubate at RT for 15 min with occasional gentle mixing

Let it stand on magnetic rack for 3 min.

Recover the supernatant.

Rinse 4X with 500 microL of 1X B&W buffer (binding and washing buffer= 5 mM Tris, pH 7.5, 0,5 mM EDTA, and 1 M NaCl) containing 1X BSA (bovine s erum albumin) wash.

Wash 2X with 200 microL of 1X ligase buffer (NEB).

【００７３】

8. Ligating linkers to bound cDNA: II linker ligation.

In this Example a linker with a recognition site for the restriction enz yme Eco RI is used. However, the invention is not dependent or limited t o the use of Eco RI in the second linker. Any other restriction enzyme a nd its recognition site can be used depending on their convenience for c loning the concatamers.

Oligonucleotides to be synthesized:

5'-GAGAGAGAGACTTTAGGTGACACTATAGAAGAGTCCTGAGAATTCNN-3'

5'-P-GAATTCTCAGGACTCTTCTATAGTGTCACCTAAAGTCTCTCTCTC-3'

The oligonucleotides are purified and annealed as described for the Linker 1.

LoTE (1 mM Tris, pH 7.5, and 0.1 mM EDTA) 20 microL suspended and add linker II (0.4 microG/microL)

Heat the tube at 65 ℃ for 5min, then let sit at room temperature for 15 min.

Add TaKaRa ligation kit II solution II 25microL and solution I 50microL.

Incubated at 16 ℃ overnight.

After ligation, wash 4 times with 500 microL 1X B&W buffer containing 1X BSA.

Wash once with 200 microL 1X B&W buffer and twice with 200 microL 1XBglII buffer containing 1X BSA.

【００７４】

Release of cDNA tags using the Tagging Enzyme

Add to the sample the following

-	LoTE	X microL
-	10X buffer	10 microL
-	Bgl II	Y microL

Make up the volume to a total of 100 microL.

1) Incubate at 37℃ for 1 hour, gently mixing intermittently.

2) Place on magnet, collect supernatant into new tube. The supernatant contains the released 5' end fragments.

3) Raise volume to 200 microL with LoTE.

To 200 microL of sample (the 5' ends, tagged with linkers) add:

133 microL 7.5M NH4Oac

3 microL 1microG/microL glycogen

340 microL Isopropanol

Incubate at 20 or -80℃ for at least 30 min.

Spin for 20min at 4℃ at 15,000 rpm in a micro-centrifuge. Remove the supernatant. Wash the pellet twice with 80% or 70% ethanol. Centrifuge for 3 min at 15,000 rpm and removed the ethanol wash. At the end, re-suspend in 10 microL LoTE.

【００７５】

Ligating tags to form di-tags

The 5' ends of cDNAs are ligated to form di-tags.

1) Add the TaKaRa ligation Kit II solution II 10 microL and solution I 20 microL.

2) Incubate overnight 16℃.

3) Added 10 microL of ddH_2O, 1 microL of 0.5M EDTA, microL of 10% SDS 1 and 1 microL of 10 microG/microL Proteinase K.

4) Incubate at 45℃ for 15min.

5) Extract once with 1:1 Tris-equilibrated phenol:chloroform aqueous phase. After phenol-chloroform and chloroform, and back extraction.

6) Removal the smallest cDNA fragment with a G-50 spun-column (Size exclusion).

7) precipitate with isopropanol by adding 5 microG of glycogen as carrier.

100 microL sample

67 microL 7.5M NH_4OAc

5 microL glycogen

180 microL Isopropanol

8) Spin for 20 min at 4 ℃.

9) Wash twice with 80% or 70% ethanol, centrifuge and remove the ethanol.

【００７６】

12. Cleavage of cDNA with anchoring enzyme

1) Re-suspend the sample in 5 microL of LoTE. Add then in order:

LoTE X microL

10X EcoRI restriction buffer 5 microL

EcoRI Y microL (use 20 Units of EcoRI)

Bring up the volume to a total of 50 microL.

2) Incubate at 37℃ for 1hour.

3) Add 1 microL of 0.5M EDTA, 1microL of 10% SDS 1 and 1 microL of 10 microG/microL Proteinase K 10%.

4) Incubate at 45 ℃ for 15min.

5) Extract once with 1:1 Tris-equilibrated phenol:chloroform aqueous phase. After phenol-chloroform and chloroform, and back extraction

6) precipitate with isopropanol by adding 5 microG of glycogen as carrier.

100 microL sample

67 microL 7.5M NH4OAc

5 microL glycogen

180 microL Isopropanol

8) Spin for 20 min at 4℃.

9) Wash twice with 80% or 70% ethanol, centrifuge and removed the ethanol wash each time.

【００７７】

11. Ligation of di-tags to form concatemers

1) Resuspended LoTE 5 microL.

2) Added TaKaRa ligation kit II solution II 5 microL and solution II 10 microL.

3) Incubate 1.5 hours at 16 ℃.

4) Added 0.5M EDTA 1 microL, 10% SDS 1 microL, 10 microG/microL Proteinase K 1 microL.

5) Incubate at 45℃ for 15min.

6) Extract once with 1:1 Tris-equilibrated phenol:chloroform aqueous phase. After phenol-chloroform and chloroform, and back extraction.

7) precipitate with isopropanol by adding 5 microG of glycogen as carrier.

100 microL sample

67 microL 7.5M NH4OAc

5 microL glycogen

180 microL Isopropanol

8) Spin for 20min at 4℃.

9) Wash twice with 80% or 70% ethanol, centrifuge and removed.

Resolved 5 microL ddH_2O.

【００７８】

Example 2:

The above-obtained concatamers are to be further ligated into a cloning vector such as pBlueascript II KS+ (Stratagene). A large variety of cloning vectors are known in the filed, which can be use for invention.

Standard Ligation:

Mix a three time excess of concatamer DNA and 100 ng of an appropriate vector linearized with Eco RI in a volume of 5 microL. Then mix 5 microL

of Solution I of DNA Ligation Kit Ver.2 (Takara) to the insert/vector mixture. Incubate the tube at 16°C for 12-16 h.

【００７９】

Transformation:

To remove salt from the ligation solution, precipitate DNA after the addition of 2 microG of Glycogen (Roche), 20mM Sodium Chloride and 80% ethanol. The DNA pellet is washed twice with 150 microL of 80% of ethanol, and the pellet is then dissolved in 10 microL of water. Using 1 microL of desalted ligation solution, ElectroMAX™ DH10B™ Cells (Invitrogen) are transformed using Cell-Porator or alike (Biometrer according to the transformation procedures described in the manufacturer's manual. Transformed bacteria are plated on a selective medium and grown overnight. Positive clones are to be isolated from those plates for further characterization of the concatamers.

【００８０】

Example 3: Sequencing of concatemers

Sequencing of concatamers is performed using primers nested in the flanking regions of the cloning vector and a BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 (Applied Biosystems) and an ABI3700 (Applied Biosystems) sequencer according to the manufacture's product descriptions. The concatamers are sequenced from both ends to cover their entire sequence.

【００８１】

Example 4: Identification of 5'-end sequence tags

The sequences obtained form Concatamers are characterized by the structure of the di-tags as presented in Figure 5. Defined regions holding the recognition sites for the restriction enzymes used during the cloning steps flank each 5' end specific sequence tag. Therefore the 5' end specific sequence tags can be identified by a manual sequence analysis or by

an automated process using an appropriate computer program. Individual 5' end specific sequence tags can be stored in a computer file or a data base system.

【００８２】

Example 5: Characterization of 5'-end sequence tags

5' end specific sequence tags can be analyzed for their identity by standard software solutions to perform sequence alignments like NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), FASTA, available in the Genetics Computer Group (GCG) package from Accelrys Inc. (http://www.accelrys.com/) or alike. Such software solutions allow for an alignment of 5' end specific sequence tags among one another to identify unique or non-redundant tags, which can be further used in

Database searches

Building a 5'-end sequence database

Gene identification using a 5'-end sequence database

An example of a BLAST search in GenBank using a 5' end specific tag is given below: The 16 bp tag (5'-ACC TCC CTC CGC GGA G) is derived from the 5' end of Human TGF-b1: JBC 264 (1989) 402-408.

Query= (16 letters)(ACCTCCCTCCGCGGAG)

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences)

1,205,903 sequences; 5,297,768,116 total letters

Score E

Sequences producing significant alignments: (bits) Value

gi|10863872|ref|NM_000660.1| Homo sapiens transforming grow... 32 1.1

gi|18590091|ref|XM_085882.1| Homo sapiens similar to transf... 32
1.1
gi|11424057|ref|XM_008912.1| Homo sapiens transforming grow... 32
1.1
gi|7684381|gb|AC011462.4|AC011462 Homo sapiens chromosome 1... 32
1.1
gi|15027087|emb|AL389894.4|LMFLCHR4A Leishmania major Fried... 32
1.1
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1.1
gi|37097|emb|X05839.1|HSTGFBG1 Human transforming growth fa... 32
1.1
gi|37092|emb|X02812.1|HSTGFB1 Human mRNA for transforming g... 32
1.1
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1.1
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gi|63465|emb|V00414.1|GGHI01 Gallus gallus mRNA coding for ... 30
4.2
gi|63444|emb|X13894.1|GGH2AF Chicken histone H2A.F gene 30
4.2

Alignments

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eta 1
(Camurati-Engelmann disease) (TGFB1), mRNA
Length = 2745

Score = 32.2 bits (16), Expect = 1.1
Identities = 16/16 (100%)
Strand = Plus / Plus

Query: 1 acctcccctccgcggag 16
||||||||||||||||
Sbjct: 1 acctcccctccgcggag 16

>gi|18590091|ref|XM_085882.1| Homo sapiens similar to transforming grow
th factor, beta 1 (H.
sapiens) (LOC147760), mRNA
Length = 697

Score = 32.2 bits (16), Expect = 1.1
Identities = 16/16 (100%)
Strand = Plus / Plus

Query: 1 acctcccctccgcggag 16
||||||||||||||||
Sbjct: 7 acctcccctccgcggag 22

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beta 1 (TGFB1), mRNA
Length = 2741

Score = 32.2 bits (16), Expect = 1.1

Identities = 16/16 (100%)

Strand = Plus / Plus

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Query: 1  acctcccctccgcggag 16
          ||||||||||||||||
Sbjct: 1  acctcccctccgcggag 16
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Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences)

Posted date: Apr 9, 2002 10:59 AM

Number of letters in database: 1,002,800,820

Number of sequences in database: 1,205,903

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1.37	0.711	1.31

Gapped

Lambda	K	H
1.37	0.711	1.31

Matrix: blastn matrix:1 -3

Gap Penalties: Existence: 5, Extension: 2

Number of Hits to DB: 6901

Number of Sequences: 1205903

Number of extensions: 6901

Number of successful extensions: 1479

Number of sequences better than 10.0: 16

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length of database: 5,297,768,116

effective HSP length: 15

effective length of query: 1

effective length of database: 5,279,679,571

effective search space: 5279679571

effective search space used: 5279679571

T: 0

A: 30

X1: 6 (11.9 bits)

X2: 15 (29.7 bits)

S1: 12 (24.3 bits)

S2: 15 (30.2 bits)

Top of Form

1: NM_000660. Homo sapiensRelated Sequences, OMIM, Protein, PubMed, tran...[gi:10863872] Taxonomy, UniSTS, LinkOut

LOCUS NM_000660 2745 bp mRNA linear PRI 13-FEB-2002

DEFINITION Homo sapiens transforming growth factor, beta 1 (Camurati-Engelmann
disease) (TGFB1), mRNA.

ACCESSION NM_000660

VERSION NM_000660.1 GI:10863872

KEYWORDS .

SOURCE human.

ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 2745)

AUTHORS　Derynck,R., Jarrett,J.A., Chen,E.Y., Eaton,D.H., Bell,J.R., Assoian,R.K., Roberts,A.B., Sporn,M.B. and Goeddel,D.V.

TITLE　Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells

JOURNAL　Nature 316 (6030), 701-705 (1985)

MEDLINE　85296301

REFERENCE　2 (bases 1 to 2745)

AUTHORS　Sporn,M.B., Roberts,A.B., Wakefield,L.M. and Assoian,R.K.

TITLE　Transforming growth factor-beta: biological function and chemical structure

JOURNAL　Science 233 (4763), 532-534 (1986)

MEDLINE　86261803

PUBMED　3487831

REFERENCE　3 (bases 1 to 2745)

AUTHORS　Chang,N.S., Mattison,J., Cao,H., Pratt,N., Zhao,Y. and Lee,C.

TITLE　Cloning and characterization of a novel transforming growth factor-beta1-induced TIAF1 protein that inhibits tumor necrosis factor cytotoxicity

JOURNAL　Biochem. Biophys. Res. Commun. 253 (3), 743-749 (1998)

MEDLINE　99119079

PUBMED　9918798

REFERENCE　4 (bases 1 to 2745)

AUTHORS　Ghadami,M., Makita,Y., Yoshida,K., Nishimura,G., Fukushima,Y., Wakui,K., Ikegawa,S., Yamada,K., Kondo,S., Niikawa,N. and To

mita,H.

TITLE Genetic mapping of the Camurati-Engelmann disease locus to chromosome 19q13.1-q13.3

JOURNAL Am. J. Hum. Genet. 66 (1), 143-147 (2000)

MEDLINE 20100617

PUBMED 10631145

REFERENCE 5 (bases 1 to 2745)

AUTHORS Vaughn,S.P., Broussard,S., Hall,C.R., Scott,A., Blanton,S.H.
,

Milunsky,J.M. and Hecht,J.T.

TITLE Confirmation of the mapping of the Camurati-Englemann locus to

19q13. 2 and refinement to a 3.2-cM region

JOURNAL Genomics 66 (1), 119-121 (2000)

MEDLINE 20304762

PUBMED 10843814

REFERENCE 6 (bases 1 to 2745)

AUTHORS Lim,J.M., Kim,J.A., Lee,J.H. and Joo,C.K.

TITLE Downregulated expression of integrin alpha6 by transforming growth

factor-beta(1) on lens epithelial cells in vitro

JOURNAL Biochem. Biophys. Res. Commun. 284 (1), 33-41 (2001)

MEDLINE 21268957

PUBMED 11374867

COMMENT PROVISIONAL REFSEQ: This record has not yet been subject to final

NCBI review. The reference sequence was derived from X02812.
1.

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gta

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Bottom of Form

Revised: October 24, 2001.

Query= (16 letters)

Database: GenBank Human EST entries

4,280,058 sequences; 2,114,234,064 total letters

Score E

Sequences producing significant alignments: (bits)

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Strand = Plus / Plus

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Strand = Plus / Plus

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Identities = 16/16 (100%)

Strand = Plus / Plus

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cDNA.

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Identities = 16/16 (100%)

Strand = Plus / Plus

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Identities = 16/16 (100%)
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Length = 888

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Identities = 16/16 (100%)
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Sbjct: 32 acctcccctccgcggag 47

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Identities = 14/14 (100%)
Strand = Plus / Plus

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Score = 28.2 bits (14), Expect = 6.4
Identities = 14/14 (100%)
Strand = Plus / Plus

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            ||||||||||||||
Sbjct: 916 acctcccctccgcgg 929
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Score = 28.2 bits (14), Expect = 6.4
Identities = 14/14 (100%)
Strand = Plus / Minus

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             ||||||||||||||
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Strand = Plus / Minus

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Identities = 14/14 (100%)
Strand = Plus / Plus

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Strand = Plus / Plus

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Strand = Plus / Minus

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Sbjct: 39 ctccctccgcggag 26

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Identities = 14/14 (100%)

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Score = 28.2 bits (14), Expect = 6.4

Identities = 14/14 (100%)

Strand = Plus / Minus

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Sbjct: 417 acctcccctccgcgg 430
```

>gi|12951460|emb|AL582959.1|AL582959 AL582959 LTI_NFL010_BC2 Homo sapiens cDNA clone CS0DL008YA12 3 prime.

```
          Length = 822

 Score = 28.2 bits (14), Expect = 6.4
 Identities = 14/14 (100%)
 Strand = Plus / Minus

Query: 2    cctcccctccgcgga 15
            ||||||||||||||
Sbjct: 533 cctcccctccgcgga 520
```

>gi|12764352|gb|BG254536.1|BG254536　602368464F1 NIH_MGC_91 Homo sapien
s cDNA clone IMAGE:4476902 5'.
　　Length = 1031

Score = 28.2 bits (14), Expect = 6.4
Identities = 14/14 (100%)
Strand = Plus / Plus

Query: 2　cctcccctccgcgga 15
　　|||||||||||||
Sbjct: 849 cctcccctccgcgga 862

>gi|12378592|gb|BF961317.1|BF961317　PM3-NN0223-111200-004-d03 NN0223 H
omo sapiens cDNA.
　　Length = 277

Score = 28.2 bits (14), Expect = 6.4
Identities = 14/14 (100%)
Strand = Plus / Minus

Query: 1　acctcccctccgcgg 14
　　|||||||||||||
Sbjct: 89 acctcccctccgcgg 76

>gi|12374538|gb|BF957263.1|BF957263　PM3-NN0223-241100-002-b08 NN0223 H
omo sapiens cDNA.
　　Length = 168

Score = 28.2 bits (14), Expect = 6.4

Identities = 14/14 (100%)
Strand = Plus / Minus

Query: 1 acctcccctccgcgg 14
||||||||||||||
Sbjct: 117 acctcccctccgcgg 104

>gi|12323114|gb|BF926150.1|BF926150 CM2-NT0193-301100-562-a12 NT0193 Homo sapiens cDNA.
Length = 417

Score = 28.2 bits (14), Expect = 6.4
Identities = 14/14 (100%)
Strand = Plus / Minus

Query: 2 cctcccctccgcgga 15
||||||||||||||
Sbjct: 268 cctcccctccgcgga 255

>gi|12259862|gb|BF869732.1|BF869732 IL3-ET0114-251000-316-A11 ET0114 Homo sapiens cDNA.
Length = 278

Score = 28.2 bits (14), Expect = 6.4
Identities = 14/14 (100%)
Strand = Plus / Minus

Query: 1 acctcccctccgcgg 14
||||||||||||||

Sbjct: 73 acctcccctccgcgg 60

>gi|12129894|gb|BF800905.1|BF800905 PM1-CI0110-201000-003-f08 CI0110 Ho
mo sapiens cDNA.
Length = 283

Score = 28.2 bits (14), Expect = 6.4
Identities = 14/14 (100%)
Strand = Plus / Plus

Query: 1 acctcccctccgcgg 14
||||||||||||||
Sbjct: 211 acctcccctccgcgg 224

>gi|12071436|gb|BF744760.1|BF744760 QV2-BT0635-311000-440-c11 BT0635 Ho
mo sapiens cDNA.
Length = 534

Score = 28.2 bits (14), Expect = 6.4
Identities = 14/14 (100%)
Strand = Plus / Plus

Query: 2 cctcccctccgcgga 15
||||||||||||||
Sbjct: 319 cctcccctccgcgga 332

>gi|11770407|gb|BE965733.2|BE965733 601659792R1 NIH_MGC_70 Homo sapiens
cDNA clone IMAGE:3896134 3'.
Length = 1336

Score = 28.2 bits (14), Expect = 6.4
Identities = 14/14 (100%)
Strand = Plus / Plus

```
Query: 1   acctccctccgcgg 14
           ||||||||||||||
Sbjct: 292 acctccctccgcgg 305
```

>gi|11766539|gb|BE963121.2|BE963121 601656923R1 NIH_MGC_67 Homo sapiens cDNA clone IMAGE:3865924 3'.
Length = 1442

Score = 28.2 bits (14), Expect = 6.4
Identities = 14/14 (100%)
Strand = Plus / Minus

```
Query: 3   ctccctccgcggag 16
           ||||||||||||||
Sbjct: 403 ctccctccgcggag 390
```

>gi|10348536|gb|BE890328.1|BE890328 601431783F1 NIH_MGC_72 Homo sapiens cDNA clone IMAGE:3916820 5'.
Length = 794

Score = 28.2 bits (14), Expect = 6.4
Identities = 14/14 (100%)
Strand = Plus / Plus

```
Query: 1   acctccctccgcgg 14
           ||||||||||||||
Sbjct: 115 acctccctccgcgg 128

>gi|10142985|gb|BE728993.1|BE728993  601562251F1 NIH_MGC_20 Homo sapiens
 cDNA clone IMAGE:3831924 5'.
          Length = 840

 Score = 28.2 bits (14), Expect = 6.4
 Identities = 14/14 (100%)
 Strand = Plus / Plus

Query: 1   acctccctccgcgg 14
           ||||||||||||||
Sbjct: 397 acctccctccgcgg 410

>gi|10095527|gb|BE707262.1|BE707262  PM1-HT0452-060700-008-e08 HT0452 Ho
mo sapiens cDNA.
          Length = 592

 Score = 28.2 bits (14), Expect = 6.4
 Identities = 14/14 (100%)
 Strand = Plus / Minus

Query: 1   acctccctccgcgg 14
           ||||||||||||||
Sbjct: 343 acctccctccgcgg 330

>gi|9772196|gb|BE543551.1|BE543551  601070523F1 NIH_MGC_12 Homo sapiens
```

cDNA clone IMAGE:3456940 5'.
Length = 1035

Score = 28.2 bits (14), Expect = 6.4
Identities = 14/14 (100%)
Strand = Plus / Plus

Query: 1 acctccctccgcgg 14
||||||||||||||
Sbjct: 332 acctccctccgcgg 345

>gi|9768571|gb|BE539926.1|BE539926 601060667F2 NIH_MGC_10 Homo sapiens
cDNA clone IMAGE:3447161 5'.
Length = 902

Score = 28.2 bits (14), Expect = 6.4
Identities = 14/14 (100%)
Strand = Plus / Plus

Query: 1 acctccctccgcgg 14
||||||||||||||
Sbjct: 411 acctccctccgcgg 424

>gi|9342607|gb|BE397242.1|BE397242 601290754F1 NIH_MGC_8 Homo sapiens c
DNA clone IMAGE:3621253 5'.
Length = 524

Score = 28.2 bits (14), Expect = 6.4
Identities = 14/14 (100%)
Strand = Plus / Plus

```
Query: 2   cctcccctccgcgga 15
           ||||||||||||||
Sbjct: 228 cctcccctccgcgga 241
```

>gi|9332870|gb|BE387505.1|BE387505 601274247F1 NIH_MGC_20 Homo sapiens cDNA clone IMAGE:3615538 5'.
Length = 637

Score = 28.2 bits (14), Expect = 6.4
Identities = 14/14 (100%)
Strand = Plus / Plus

```
Query: 1   acctcccctccgcgg 14
           ||||||||||||||
Sbjct: 422 acctcccctccgcgg 435
```

>gi|8140649|gb|AW950985.1|AW950985 EST363055 MAGE resequences, MAGA Homo sapiens cDNA.
Length = 638

Score = 28.2 bits (14), Expect = 6.4
Identities = 14/14 (100%)
Strand = Plus / Plus

Query: 2 cctcccctccgcgga 15

||||||||||||||

Sbjct: 273 cctcccctccgcgga 286

>gi|8139665|gb|AW950129.1|AW950129 EST362094 MAGE resequences, MAGA Homo sapiens cDNA.

Length = 611

Score = 28.2 bits (14), Expect = 6.4

Identities = 14/14 (100%)

Strand = Plus / Plus

Query: 2 cctcccctccgcgga 15

||||||||||||||

Sbjct: 273 cctcccctccgcgga 286

Database: GenBank Human EST entries

Posted date: Mar 29, 2002 2:35 AM

Number of letters in database: 2,114,234,064

Number of sequences in database: 4,280,058

Lambda	K	H
1.37	0.711	1.31

Gapped

Lambda	K	H
1.37	0.711	1.31

Matrix: blastn matrix:1 -3

Gap Penalties: Existence: 5, Extension: 2

Number of Hits to DB: 5013

Number of Sequences: 4280058

Number of extensions: 5013

Number of successful extensions: 5013

Number of sequences better than 10.0: 61

length of query: 16

length of database: 2,114,234,064

effective HSP length: 15

effective length of query: 1

effective length of database: 2,050,033,194

effective search space: 2050033194

effective search space used: 2050033194

T: 0

A: 30

X1: 6 (11.9 bits)

X2: 15 (29.7 bits)

S1: 12 (24.3 bits)

S2: 14 (28.2 bits)

Top of Form

Taxono
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1: BM915385. AGENCOURT_6701642...[gi:19365764]

IDENTIFIERS

dbEST Id: 11598757

EST name: AGENCOURT_6701642

GenBank Acc: BM915385

GenBank gi: 19365764

CLONE INFO

Clone Id: IMAGE:5481560 (5')

Plate: LLCM2006 Row: d Column: 09

DNA type: cDNA

PRIMERS

PolyA Tail: Unknown

SEQUENCE

CGCCCTGGGCCATCTCCCTCCCACCTCCCTCCGCGGAGCAGCCAGACAGCGAGGGC

CCCG

GCCGGGGGCAGGGGGGACGCCCCGTCCGGGGCACCCCCCCGGCTCTGAGCCGCCCG

CGGG

GCCGGCCTCGGCCCGGAGCGGAGGAAGGAGTCGCCGAGGAGCAGCCTGAGGCCCCA

GAGT

CTGAGACGAGCCGCCGCCGCCCCCGCCACTGCGGGGAGGAGGGGGAGGAGGAGCGG

GAGG

AGGGACGAGCTGGTCGGGAGAAGAGGAAAAAAACTTTTGAGACTTTTCCGTTGCCG

CTGG

GAGCCGGAGGCGCGGGGACCTCTTGGCGCGACGCTGCCCCGCGAGGAGGCAGGACT

TGGG

GACCCCAGACCGCCTCCCTTTGCCGCCGGGGACGCTTGCTCCCTCCCTGCCCCCTA

CACG

GCGTCCCTCAGGCGCCCCCATTCCGGACCAGCCCTCGGGAGTCGCCGACCCGGCCT

CTCG

CAAAGACTTTTCACCATACCTCGGGCGCACCCTCTGCACGCGGCCTTCATCACCGG

CCTG

TCTACTGAGCCCCCGCGGATGCCTAGACCCTTTCTCCTCCGGGAGACGGATCCCTC

TCCG

ACCTGCCGCAAATTCCCTATTCTGGAACACCCCCGCTTCCTGGGACCCTAATCCCC

GCCT

TTCGACGCTCCTTGCGCTGGGGAACTGAAGAGCCCCCGGGTTCGTAACCTTTTCCT

TCCC

CGTTTTGAAAAACATCCCCCGTTAATAAACCTTGACTATTTTCGCTTTGGGCCCCC

CCCT

TACGGTTTTTGGCGGGCACTAAACAAACATCGAGTCTCAAGGCGGCGGATGCCACT

CAAG

CCTGAATACTTTTGCGCGTTAGGGGCGGTCTTTTACGCGAGTAGAGTCGGGCCTTG

ACCG

GACCCTATTCATTGGTTTCCCGTGACGTGTGCGGGCGTAACGAGATATTAACCTCT

CCCG

ACACATTGTCATAAAACACCACTTTCGACACGCCCTACTCCTGTTAATAGTCGCCC

CCTC

CCCGCGTGTAAAATTTCCCGCGCCAATGCCCTCCATTATTCCGCTCCATGAAAAAG

GGGG

TCGGCN

Quality: High quality sequence stops at base: 467

Entry Created: Mar 11 2002

Last Updated: Mar 12 2002

COMMENTS

Tissue Procurement: DCTD/DTP

cDNA Library Preparation: Rubin Laboratory

cDNA Library Arrayed by: The I.M.A.G.E. Consortium (LLNL

)

DNA Sequencing by: Agencourt Bioscience Corporation

Clone distribution: MGC clone distribution information c

an

be found through the I.M.A.G.E. Consortium/LLNL at:

http://image.llnl.gov

LIBRARY

Lib Name: NIH_MGC_41

Organism: Homo sapiens

Organ: skin

Tissue type: amelanotic melanoma, cell line

Lab host: DH10B (phage-resistant)

Vector: pOTB7

R. Site 1: XhoI

R. Site 2: EcoRI

Description: cDNA made by oligo-dT priming. Directionally cloned into EcoRI/XhoI sites using the following 5' adaptor: GGCACGAG(G). Library constructed by Ling Hong in the laboratory of Gerald M. Rubin (University of California, Berkeley) using ZAP-cDNA synthesis kit (Stratagene) and Superscript II RT (Life Technologies). Note: this is a NIH_MGC Library.

SUBMITTER

Name: Robert Strausberg, Ph.D.

E-mail: cgapbs-r@mail.nih.gov

CITATIONS

Title: National Institutes of Health, Mammalian Gene Collection (MGC)

Authors: NIH-MGC http://mgc.nci.nih.gov/

Year: 1999

Status: Unpublished

Bottom of Form

Revised: October 24, 2001.

Check on Est in Genbank:

Query= (1086 letters)

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences)

1,205,903 sequences; 5,297,768,116 total letters

Score E

Sequences producing significant alignments: (bits) Value

gi|10863872|ref|NM_000660.1| Homo sapiens transforming grow... 587 e-165

gi|18590091|ref|XM_085882.1| Homo sapiens similar to transf... 587 e-165

gi|11424057|ref|XM_008912.1| Homo sapiens transforming grow... 587 e-165

gi|7684381|gb|AC011462.4|AC011462 Homo sapiens chromosome 1... 587 e-165

gi|37097|emb|X05839.1|HSTGFBG1 Human transforming growth fa... 587 e-165

gi|37092|emb|X02812.1|HSTGFB1 Human mRNA for transforming g... 587 e-165

gi|340526|gb|J04431.1|HUMTGFB1PR Homo sapiens transforming ... 587 e-165

gi|12654682|gb|BC001180.1|BC001180 Homo sapiens, Similar to... 291 8e-76

gi|12652748|gb|BC000125.1|BC000125 Homo sapiens, Similar to... 291
8e-76
gi|18490115|gb|BC022242.1| Homo sapiens, clone MGC:22008 IM... 153
4e-34
gi|755044|gb|M23703.1|PIGTGFB1A Sus scrofa transforming gro... 129
6e-27
gi|7650477|gb|AF249327.1|AF249327 Rattus norvegicus TGF-bet... 66
8e-08
gi|4416081|gb|AF105069.1|AF105069 Rattus norvegicus transfo... 66
8e-08
gi|2394170|gb|AF015683.1|AF015683 Rattus norvegicus transfo... 66
8e-08
gi|6755774|ref|NM_011577.1| Mus musculus transforming growt... 64
3e-07
gi|1161133|gb|L42456.1|MUSTGF1G01 Mus musculus TGF-1 gene, ... 64
3e-07
gi|3688423|emb|AJ009862.1|MMU009862 Mus musculus mRNA for t... 64
3e-07
gi|201947|gb|M57902.1|MUSTGFB1 Mouse transforming growth fa... 64
3e-07
gi|18042365|gb|AC097483.3| Homo sapiens BAC clone RP11-146N... 44
0.30
gi|17481821|ref|XM_008785.3| Homo sapiens one cut domain, f... 44
0.30
gi|12737997|ref|XM_007116.2| Homo sapiens Zic family member... 44
0.30
gi|6005961|ref|NM_007129.1| Homo sapiens Zic family member ... 44
0.30
gi|11065969|gb|AF193855.1|AF193855 Homo sapiens zinc finger... 44

0.30

gi|4758847|ref|NM_004852.1| Homo sapiens one cut domain, fa... 44

0.30

gi|15787728|emb|AL355338.33|AL355338 Human DNA sequence fro... 44

0.30

gi|4028591|gb|AF104902.1|AF104902 Homo sapiens ZIC2 protein... 44

0.30

gi|1531593|gb|U50523.1|HSU50523 Human BRCA2 region, mRNA se... 44

0.30

gi|4468940|emb|Y18198.1|HSAY18198 Homo sapiens mRNA for ONE... 44

0.30

gi|19067958|gb|AY049805.1| Alopias pelagicus 5.8S ribosomal... 42

1.2

gi|18025465|gb|AY037858.1| Cercopithicine herpesvirus 15 st... 42

1.2

gi|12039248|gb|AC020659.5|AC020659 Homo sapiens chromosome ... 42

1.2

gi|19909461|gb|AC098709.3| Mus musculus clone RP23-1K14, co... 40

4.6

gi|19921137|ref|NM_135651.1| Drosophila melanogaster (CG47... 40

4.6

gi|18376846|gb|AC092198.2| Homo sapiens chromosome X clone ... 40

4.6

gi|18467841|ref|XM_078995.1| CG4751 (CG4751), mRNA 40

4.6

gi|18376869|gb|AC091898.2| Homo sapiens chromosome 5 clone ... 40

4.6

gi|18030132|gb|AC026695.5| Homo sapiens chromosome 5 clone ... 40

4.6

gi|15887302|gb|AC020914.8|　Homo sapiens chromosome 19 clone...　40
4.6
gi|14578122|gb|AC092241.1|AC092241　Drosophila melanogaster,...　40
4.6
gi|15292266|gb|AY051978.1|　Drosophila melanogaster LD44770 ...　40
4.6
gi|15055218|gb|AC060226.39|　Homo sapiens 12 BAC RP11-101P14...　40
4.6
gi|14389338|gb|AC084282.6|AC084282　Oryza sativa chromosome ...　40
4.6
gi|13677167|gb|AC015977.9|AC015977　Homo sapiens clone RP11-...　40
4.6
gi|9910225|ref|NM_020179.1|　Homo sapiens FN5 protein (FN5),...　40
4.6
gi|10440613|gb|AC069145.5|AC069145　Oryza sativa chromosome ...　40
4.6
gi|10728714|gb|AE003631.2|AE003631　Drosophila melanogaster ...　40
4.6
gi|9246422|gb|AF197137.1|AF197137　Homo sapiens FN5 protein ...　40
4.6
gi|4190938|gb|AC000091.1|AC000091　Homo sapiens Chromosome 2...　40
4.6
gi|17431932|emb|AL646085.1|AL646085　Ralstonia solanacearum ...　40
4.6
gi|15073719|emb|AL591785.1|SME591785　Sinorhizobium meliloti...　40
4.6
gi|3628578|gb|AC005115.1|AC005115　Drosophila melanogaster D...　40
4.6
gi|3150432|gb|U50080.1|LSU50080　Lymnaea stagnalis serotonin...　40

4.6

gi|8052359|emb|AL356592.1|SC9H11 Streptomyces coelicolor co... 40

4.6

gi|6624640|emb|AL034344.24|HS118B18 Human DNA sequence from... 40

4.6

gi|15528721|dbj|AP003296.3| Oryza sativa (japonica cultivar... 40

4.6

gi|15289781|dbj|AP003141.2| Oryza sativa (japonica cultivar... 40

4.6

gi|6069643|dbj|AP000616.1| Oryza sativa (japonica cultivar-... 40

4.6

gi|960285|gb|L46862.1|RATLAMB2G Rattus norvegicus laminin B... 40

4.6

gi|198704|gb|J03749.1|MUSLAMB2B Mouse laminin B2 gene, exon... 40

4.6

gi|198702|gb|J02930.1|MUSLAMB2A Mouse laminin B2 chain mRNA... 40

4.6

gi|198694|gb|J03484.1|MUSLAM2B Mouse laminin B2 chain mRNA,... 40

4.6

Alignments

>gi|10863872|ref|NM_000660.1| Homo sapiens transforming growth factor, beta 1 (Camurati-Engelmann
disease) (TGFB1), mRNA

Length = 2745

Score = 587 bits (296), Expect = e-165

Identities = 356/377 (94%), Gaps = 1/377 (0%)

Strand = Plus / Plus

```
Query: 246 cgagctggtcgggagaagaggnnnnnnnctttgagacttttccgttgccgctgggagcc
305
           ||||||||||||||||||||      ||||||||||||||||||||||||||||||||
Sbjct: 225 cgagctggtcgggagaagaggaaaaaaactttgagacttttccgttgccgctgggagcc
284

Query: 306 ggaggcgcggggacctcttggcgcgacgctgccccgcgaggaggcaggacttggggaccc
365
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 285 ggaggcgcggggacctcttggcgcgacgctgccccgcgaggaggcaggacttggggaccc
344

Query: 366 cagaccgcctccctttgccgccggggacgcttgctccctccctgccccctacacggcgtc
425
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 345 cagaccgcctccctttgccgccggggacgcttgctccctccctgccccctacacggcgtc
404

Query: 426 cctcaggcgcccccattccggaccagccctcgggagtcgccgacccggcctctcgcaaag
485
           |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct: 405 cctcaggcgcccccattccggaccagccctcgggagtcgccgacccggcctcccgcaaag
464

Query: 486 acttttcaccatacctcgggcgcaccctctgcacgcggccttcatcaccggcctgtctac
545
           ||||||| ||| |||||||||||||| |||||||| ||||||||| |||||||||| |
Sbjct: 465 acttttccccagacctcgggcgcacccccctgcacgccgccttcatccccggcctgtctcc
524
```

Query: 546 tgagcccccgcggatgcctagacccttttctcctccgggagacggatccctctccgacctg
605

Sbjct: 525 tgagcccccgcgcat-cctagacccttttctcctccaggagacggatctctctccgacctg
583

Query: 606 ccgcaaattccctattc 622

Sbjct: 584 ccacagatcccctattc 600

【００８３】

Example 6: Statistical analysis of 5' end sequence tags

5' end sequence tags obtained from the same plurality of mRNAs in a sample or nucleic acid fragments within the same cDNA library can be analyzed by a standard software solution like NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to identify non-redundant sequence tags as describe in Example 5. All such non-redundant sequence tags can then be individually counted and further analyzed for the contribution of each non-redundant tag to the total number of all tags obtained from the same sample. The contribution of an individual tag to the total number of all tags should allow for a quantification of the transcripts in a plurality of mRNAs in the sample or a cDNA library. The results obtained in such a way on individual samples can be further compared with similar data obtained from other samples to compare their expression patterns.

【００８４】

Example 7: Mapping of 5' end sequence tags to genomic sequence information

5' end specific sequence tags obtained as describe in this Example can be used to identify transcribed regions within genomes for which partia

l or entire sequences were obtained. Such a search can be performed usin g standard software solutions like NCBI BLAST (http://www.ncbi.nlm.nih.g ov/BLAST/) to align the 5' end specific sequence tags to genomic sequenc es. In the case of large genomes like those from human, rat or mouse it may be necessary to extend the initial sequence information obtained fro m concatemers for example by the approach describe in Example #. The use of extended sequences allows for a more precise identification of activ ely transcribed regions in the genome.

【００８５】

Example 8: Identification of transcriptional start sites

5' end specific sequence tags, which could be mapped to genomic sequen ces, allow for the identification of regulatory sequences. In a gene the DNA upstream of the 5' end of transcripted regions usually encompasses most of the regulatory elements, which are used in the control of gene e xpression. These regulatory sequences can be further analyzed for their functionality by searches in databases, which hold information on bindin g sites for transcription factors. Publicly available databases on trans cription factor binding sites and for promoter analysis include:

Transcription Regulatory Region Database (TRRD) (http://wwwmgs.bionet.ns c.ru/mgs/dbases/trrd4/)

TRANSFAC (http://transfac.gbf.de/TRANSFAC/)

TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html)

PromoterInspector provide by Genomatix Software (http://www.genomatix.de /)

【００８６】

Example 9: Cloning of full-length cDNAs using information derived from 5 ' end sequence tags

Sequence information derived from the concatamers can be used to synth

esis specific primers for the cloning of full-length cDNAs. In such an approach, the sequence derived from a given 5' end specific tag can be used to design a forward primer while the choice of the reverse primer would be dependent on the template DNA used in the amplification reaction. Amplification by the polymerase chain reaction (PCR) can be performed using a template derived from a plurality of RNA obtained from a biological sample and an oligo-dT primer. In the first step the oligo-dT primer and a reverse transcriptase are used to synthesis a cDNA pool. In the second step a forward primer derived from a 5' end specific tag and an oligo-dT primer are used to amplify a full-length cDNA from the cDNA pool. Similarly, a specific full-length cDNA can be amplified from an exiting cDNA library using a forward primer derived from a 5' end tag and a vector nested reversed primer.

【００８７】

Example 10: Alternative approaches for the cloning of 5'-end tags from cDNA libraries.

A plurality of cDNAs can be amplified from an exciting cDNA library having a recognition site for a class IIs endonuclease at the 5' end of the inserts. The PCR products derived from such a library would be further treated as described in the examples herein.

【００８８】

Example 11: Cloning of 5' ends by replacement of the Cap structure by an oligonucleotide having a class IIs recognition site

A cDNA/RNA hybrid encompassing the 5' end of an initial transcript can be obtained as described in Example 1. The Cap structure in such cDNA/RNA hybrids is then enzymatically removed by a hydrolyzing enzyme such as the T4 polynucleotide kinase or the tobacco acid pyrophosphatase. A single or double stranded oligonucleotide having a class IIs recognition site is then ligated by T4 RNA ligase to the RNA at the phosphate present

at the 5' end of the de-capped mRNA. The ligated oligonucleotide will fu nction as a primer for the second strand synthesis following the procedu re given in Example 1. By the use of a modified oligonucleotide in the l igation step the double stranded cDNA can be attached to a support and u sed for the cloning of concatamers as described herein.

【００８９】

Example 12: Amplification step for a sample

In cases where the amount of a sample is limiting to the invention, th e sample material can be amplified by the following approach. In a first step a plurality of mRNAs is treated as described in Example 11 to repl ace the cap structure by an appropriate oligonucleotide having a class I Is recognition site. In a second step the aforementioned template is amp lified by a PCR step using a primer complementary to the linker and a po ly-A primer. The PCR product can be used for the invention as described in the Examples 1.

【００９０】

Example 13: Utilization of extended 5'-end sequences

Initial 5' end sequences obtained for concatamers can be used to synth esis sequencing primers to obtain extended sequence information on the 5 ' end of a transripted region.

【００９１】

Example 14: Gene inactivation

Sequence information obtained from 5' end specific sequence tags can be used for the design of anti-sense probes, which could be applied in knoc kdown studies.

【００９２】

【発明の効果】

By the present invention, novel means by which not only the informatio n on the nucleotide sequences of mRNAs contained in a sample may be obta

ined, but also novel genes could be cloned. By the method of the present invention, information on the nucleotide sequences of the 5' end regions of a plurality of nucleic acids said mRNAs and cDNAs in the sample could effectively be obtained. Since the information on the nucleotide sequences of the 5' end regions is obtained, unknown genes can be cloned after the identification of a novel transcript. Further, it may be possible to attain mapping of transcription start sites, mapping of promoter usage pattern, analysis of SNPs in promoters, creating gene networking by combining the expression analysis, alternative promoter usage and the other data in this disclosure, and selective recovery of promoter regions in fragmented genomic DNA.

【００９３】

In particular, the invention has a great impact on identification, cloning and further analysis of promoter regions. After sequencing concatamer libraries holding information on a plurality of 5' ends, a statistical analysis on the distribution on the transcriptional start sites will be possible. Changes between different physiological conditions switch the mRNA transcription machinery into new "status". Such a "transcriptional status" can measured by computing (1) the presence of the transcription starting points, (2) the digital expression of the various transcriptional factors by counting their expression by counting the tags, and correlating the presence of starting point, the transcription factors. More information will be obtained on the gene networking by comparing the perturbation of gene expression between two different conditions. Such comparisons of transcriptional conditions between various disease and normal tissues could allow for the design of new and very comprehensive diagnostic tools. Thus the invention will be of high commercial value in gene discovery and gene analysis, and it is envisioned that the invention will be of use in the development of novel diagnostic and therapeutic produ

cts.

【００９４】

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4 Brief Description of Drawings

【図１】

An example for the preparation of a plurality of 1st strand cDNAs is pr esented, where the starting material can be RNA derived from a biologica l sample or a cDNA library.

【図２】

An example for the cloning of 5' -end specific tags into concatemers is presented. The example is including but not limited to the use of the r estriction enzymes Gsu I, Bgl II and Eco RI.

【図３】

An example for a 1st linker to be used for the cloning of 5' -end speci

fic tags is presented. The example is including but not limited to the use of the restriction enzymes Bgl II, Gsu I, and Mme I.

【図４】

An example for a 2nd linker to be used for the cloning of 5' -end specific di-tags is presented. The example is including but not limited to the use of the restriction enzymes Bgl II, Gsu I, Mme I and Eco RI.

【図５】

An example for the structure of a di-tag is presented. The example is including but not limited to the use of the restriction enzymes Bgl II, Gsu I, Mme I and Eco RI.

【図６】

An example for the use of a 5' -end specific linker is presented, in which the linker is used for the enrichment of individual nucleic acids and their sequencing.

【書類名】　外国語図面

【図１】

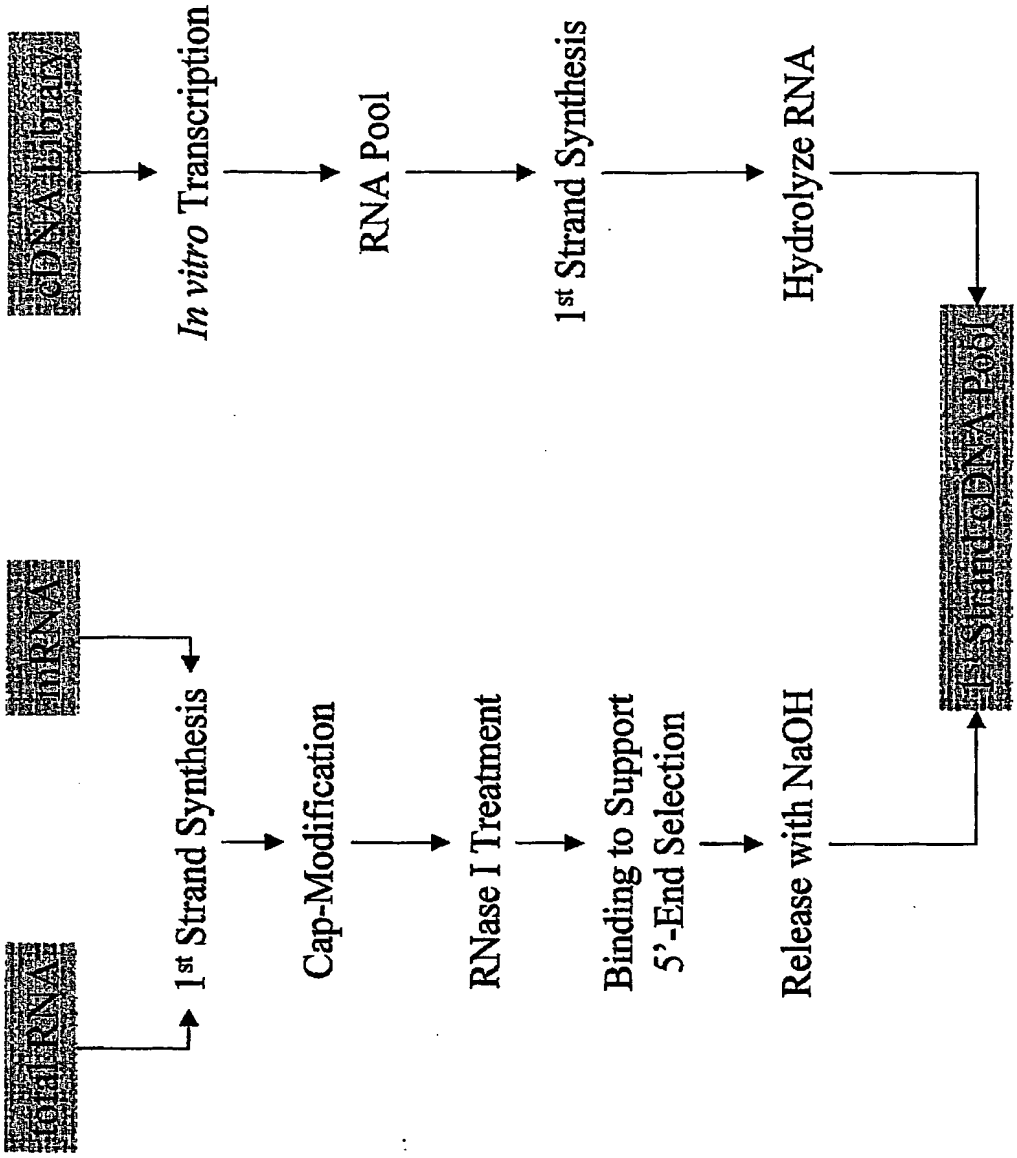


Fig.1: 1st Strand Synthesis

【図２】

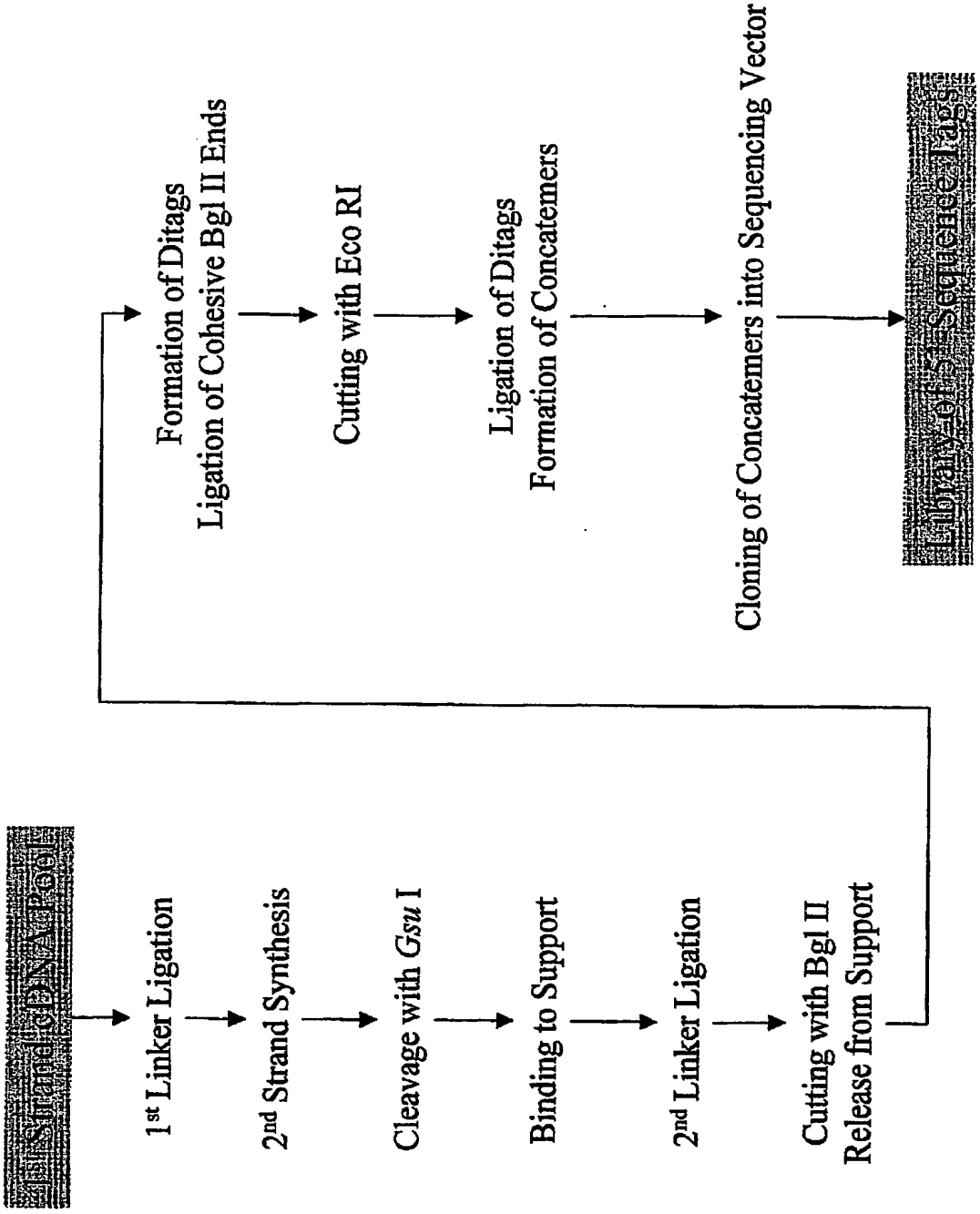
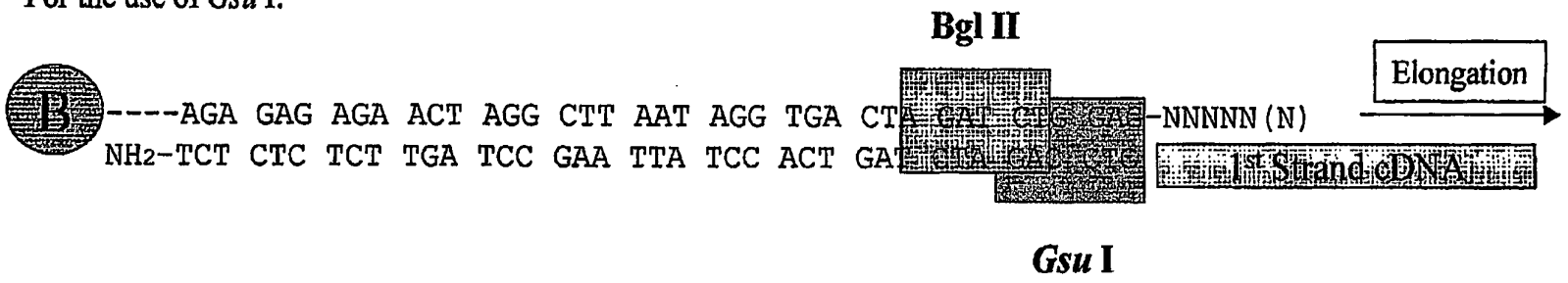


Fig.2: Cloning of Concatemers

For the use of *Gsu* I:



For the use of *Mme* I:

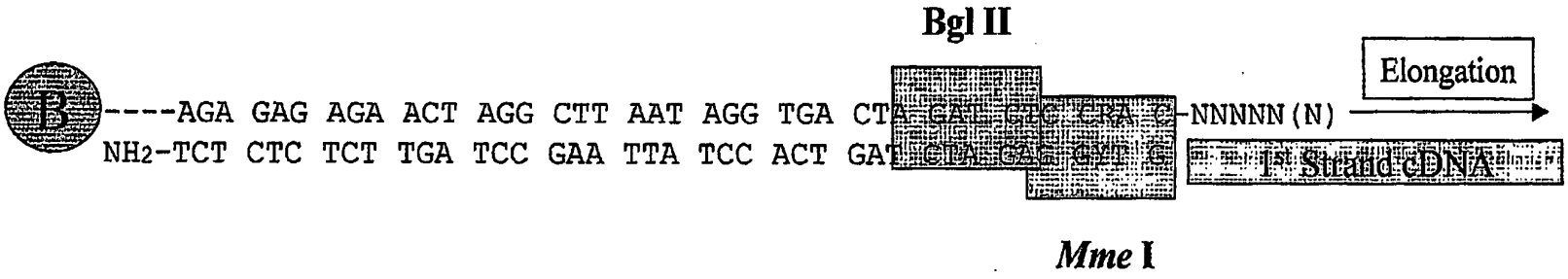
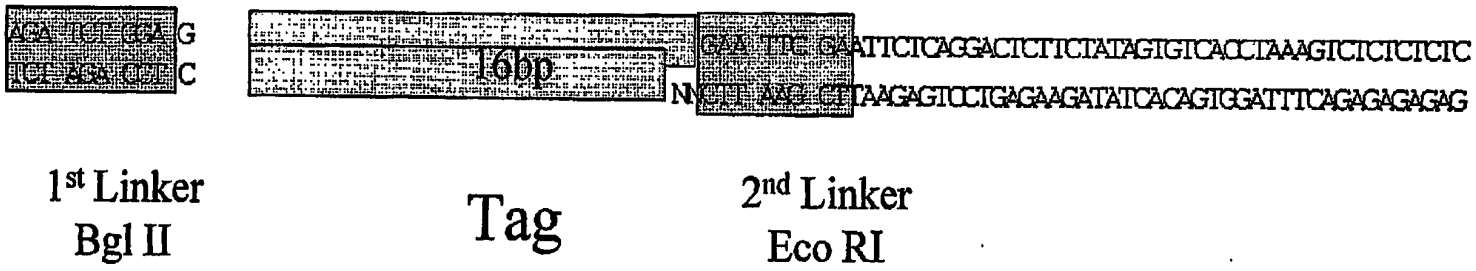


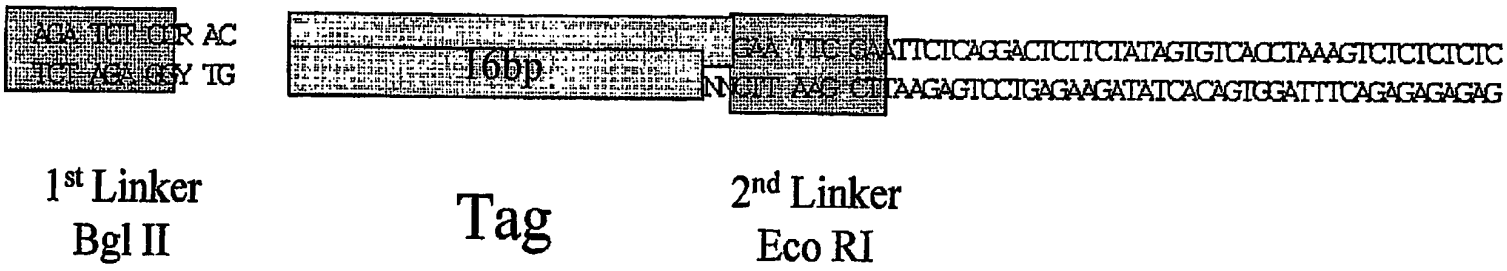
Fig.3: 1st Linker Ligation

【図３】

For the use of *Gsu* I:



For the use of *Mme* I:



R = G or A
Y = C or T

Fig.4: 2nd Linker Ligation

【図５】

Fig.5: Structure of a Di-tag

【図６】

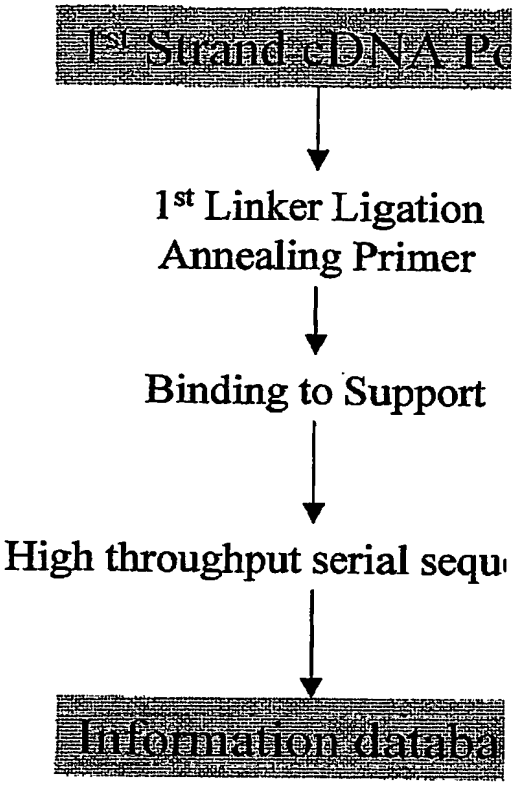


Fig.6: Serial Sequencing

【書類名】　外国語要約書

1　Abstract

A method is disclosed to obtain the 5' ends of transcribed regions from a plurality of nucleic acid fragments obtained from biological materials or synthetic pools. DNA fragments encoding the 5' ends are enriched for their individual analysis or for the analysis of concatamers thereof. The sequence information derived from 5' ends can be used for characterization and cloning of the transcriptome.

2　Representative Drawing　None

認定・付加情報

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